POLIOVIRUS RNA POLYMERASE: ISOLATION AND CHARACTERIZATION OF THE POLYMERASE AND ITS *IN VITRO* RNA PRODUCT

BY

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To my family.

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ABBREVIATIONS USED

ATP.....adenosine-5'-triphosphate

BSA.....bovine serum albumin

CTP.....cytidine-5'-triphosphate

d.....dalton

DEP.....diethylpyrocarbonate

DNA.....deoxyribonucleic acid

ds.....double-stranded

DTT.....dithiothreitol

EDTA.....ethylenediaminetetraacetic acid

EMCV.....encephalomyocarditis virus

FMDV.....foot-and-mouth disease virus

g.....gravity

GMP.....guanosine-5'-monophosphate

GTP.....guanosine-5'-triphosphate

HEPES.....N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

MEM.....minimum essential medium

moi.....multiplicity of infection

Mr.....relative molecular weight

mRNA.....messenger ribonucleic acid

NCVP.....noncapsid viral protein

NTP.....nucleoside triphosphate

oligo(U).....oligouridylic acid

PFU.....plaque-forming units

PIPES.....piperazine-N,N'-bis(2-ethanesulfonic acid)

poly(A).....polyadenylic acid

RF.....replicative form

RI.....replicative intermediate

RNA.....ribonucleic acid

RNase.....ribonuclease

S.....Svedberg (unit of sedimentation)

SDS.....sodium dodecyl sulfate

ss.....single-stranded

TES.....2-{[tris-(hydroxylmethyl)methyl]amino}
ethanesulfonic acid

UMP.....uridine-5'-monophosphate

UTP.....uridine-5'-triphosphate

UV.....ultraviolet

V.....volts

VP.....viral protein

VPg.....viral protein, genome-linked

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A soluble RNA-dependent RNA polymerase was isolated from poliovirus-infected HeLa cells and was shown to copy poliovirion RNA *in vitro*. The polymerase was purified from a 200,000 x g supernatant of a cytoplasmic extract of infected cells by ammonium sulfate precipitation, phosphocellulose chromatography, gel filtration on Sephacryl S-200, and hydroxylapatite chromatography. The activity of the enzyme was assayed throughout the purification by using a polyadenylic acid [poly(A)] template and an oligouridylic acid [oligo(U)] primer. The polymerase precipitated in a solution that was 35% saturated with ammonium sulfate and eluted from a phosphocellulose column when the KCl concentration reached 0.15 M. These two purification steps resulted in about an eightyfold purification of the enzyme relative to the high speed supernatant. The polymerase was purified further by gel filtration in Sephacryl S-200 and by hydroxylapatite

chromatography. The polymerase eluted from a hydroxylapatite column between 0.25 M and 0.5 M potassium phosphate and, after dialysis, was stored at -70°C for up to six months with little loss in activity.

The purified polymerase was free of detectable ribonuclease activity and was able to copy poliovirion RNA in the presence of oligo(U), all four ribonucleoside triphosphates, and Mg^{2+}. The optimal molar ratio of oligo(U) to virion RNA was about 8:1. The Mg^{2+} concentration significantly affected the activity of the enzyme, with incorporation of labeled substrate into product RNA being maximal at 3 mM $Mg(CH_3COO)_2$. Polymerase activity was inhibited by heparin, EDTA, 1 mM Mn^{2+}, and any increase in the KCl concentration.

The polypeptide composition of the purified polymerase was determined by isolating the polymerase from infected HeLa cells which had been labeled with [^{35}S]methionine and by analyzing the polypeptides present using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A single virus-specific protein that had an apparent molecular weight of 63,000 (p63) was found to copurify with polymerase activity. Two-dimensional gel electrophoresis of the polymerase protein indicated that it had an isoelectric point of about 7.5. No host-coded proteins were recovered in equimolar amounts relative to p63. Noncapsid viral protein 2 (NCVP2) and other viral proteins were clearly separated from p63 by Sephacryl S-200 chromatography. Under the reaction conditions tested NCVP2 was totally inactive as an RNA polymerase and did not stimulate the polymerase activity of p63.

When virion RNA was used as a template in the in vitro reaction, the purified polymerase synthesized labeled product RNA which was shown to be full-sized by CH_3HgOH-agarose gel electrophoresis. A

nearest-neighbor analysis of the full-sized product RNA showed that it was heteropolymeric. The product RNA was resistant to RNase digestion when annealed with excess virion RNA and thus appeared to be a true complementary copy of the virion RNA template. In addition, the product RNA was shown to be covalently linked to the oligo(U) used in the reaction as primer.

Initiation and elongation rates in the in vitro reaction were found to be significantly affected by changes in the pH, temperature, and Mg^{2+} concentration. Optimum conditions for initiation and elongation were not the same. Under optimal conditions for elongation [HEPES buffer (pH 8.0), 7 mM Mg^{2+}, 37°C], full-sized product RNA was synthesized in about six minutes. Under these conditions, most of the product RNA synthesized in a one-hour reaction was full-sized.

Thus, a soluble RNA-dependent RNA polymerase has been purified from poliovirus-infected cells. A single viral polypeptide, p63, appears to be responsible for this activity. This polymerase can synthesize full-length complementary copies of virion RNA in vitro using oligo(U) as a primer.

CHAPTER I
INTRODUCTION

The picornaviridae family consists of a large number of small, nonenveloped RNA viruses, many of which are human pathogens. Included in this group are the human enteroviruses (coxsackie, echo, and polio), the human rhinoviruses, and apparently hepatitis A virus. These viruses cause a wide range of human disorders such as myocarditis, aseptic meningitis, poliomyelitis, infectious hepatitis and a variety of respiratory diseases including the common cold. In addition to the human viruses, the picornavirus group contains a number of other important mammalian viruses. Among the best studied are the cardioviruses [encephalomyocarditis (EMC) virus and mengovirus] and the foot-and-mouth disease virus (FMDV). The FMD viruses infect cattle and, therefore, are of significant economic importance to the livestock industry. All of these viruses appear to be similar in their molecular structure and in their general mode of viral replication. Although numerous studies have been devoted to the picornaviruses, few details are known about the molecular mechanism by which these viruses replicate their genomic RNA. This study addresses the problem of RNA replication using the poliovirus system.

Virion Structure and Replication

The general features of the structure and replication of poliovirus are characteristic of most of the picornaviruses and are summarized in many review articles (Baltimore, 1969; Levintow, 1974; Rekosh, 1977; Sangar, 1979; Scraba, 1979; Agol, 1980).

The poliovirion consists of four different polypeptides that surround a molecule of single-stranded ribonucleic acid. Each of the four capsid proteins (VP1, VP2, VP3, and VP4) is present in 60 copies per virion and the resulting arrangement forms an icosahedron. The virus has a molecular mass of 8.6×10^6 daltons, a sedimentation coefficient of 150 S and a diameter of 28-30 nm. The virion RNA ($Mr = 2.6 \times 10^6$) is of the positive polarity and is itself infectious. It has a poly(A) sequence of heterogeneous size (50-125 nucleotides) at its 3' terminus and a small protein, VPg, covalently bound to its 5' end. The typical eukaryotic m^7G5'ppp5'N(m)pNp "capping" group is not found on any intracellular viral RNA, nor does the RNA contain methylated or unusual nucleotides.

Adsorption of poliovirus to the host cell is mediated by specific receptors on the cell surface. After penetration there is an eclipse period of 2-3 hours before infectious virus can be recovered from the cell. During this time host protein, DNA, and RNA syntheses are inhibited, and the majority of macromolecular synthesis within the cell becomes virus-specific. The parental RNA serves as a messenger RNA to direct the synthesis of a large polyprotein which apparently represents most of the coding capacity of the viral genome. The primary cleavage of this polyprotein takes

place on the ribosomes, presumably by host proteases, and yields a precursor to the capsid proteins as well as two noncapsid protein precursors. Subsequently, numerous proteolytic cleavages occur which yield the spectrum of viral polypeptides.

Translation of the parental RNA is a prerequisite for viral RNA replication, and this suggests that one or more of the components of the RNA-dependent RNA polymerase present in infected cells is coded for by the virus. Once translated, this RNA serves as a template for the synthesis of a complementary minus-strand RNA, from which messenger and virion RNA are transcribed. Ribonucleic acid synthesis, which is associated with host cell membranes, is semiconservative and asymmetric (greater than 90% of the RNA synthesized is of the positive polarity). The plus strands either serve as message or immediately become incorporated into procapsids containing the polypeptides VP0, VP1 and VP3. In situ cleavage of VP0 to VP2 and VP4 effects the final maturation of the virion. The replication cycle is completed with degeneration of the host cell and the release of infectious virus approximately seven hours after infection.

Synthesis of Viral Proteins

Two features of poliovirus replication have greatly aided in the study of virus-specific protein synthesis and processing. First is the shut off of host protein synthesis, which allows the selective incorporation of radiolabeled amino acids into viral proteins. The second is the presence of one major initiation site for the translation of a single virus message. This has facilitated the

determination of a map order for the poliovirus polypeptides. The following is a synopsis of results from many studies in which these features were utilized to study poliovirus protein synthesis *in vivo*.

The largest poliovirus protein (NCVP00) has an apparent molecular weight of approximately 2.1×10^5 and is the precursor to most, if not all, of the poliovirus proteins (Rueckert, *et al.* 1979). It has been synthesized *in vitro* (Villa-Komaroff *et al.*, 1975), but can only be detected in infected cells when peptide cleavage is inhibited (Jacobson and Baltimore, 1968). Normally, NCVP00 is cleaved as a nascent polypeptide to yield three primary products. In order from the amino terminus they are NCVP1a, NCVPX, and NCVP1b (Taber, *et al.* 1971 and Summers and Maizel, 1971). Several subsequent cleavages of these proteins, probably by virus-coded proteases (Korant, 1979), yield the rest of the viral proteins. The precursor-product relationships have been established by pulse-chase experiments and by tryptic peptide map analysis (reviewed by Rueckert *et al.*, 1979). Capsid proteins (VP1, Mr ∿ 34,000; VP2, Mr ∿ 30,000; VP3, Mr ∿ 25,000; and VP4, Mr ∿ 4,000-8,000) are encoded in the 5' half of the genome and result from the cleavage of NCVP1a (Mr ∿ 95,000). The rest of the proteins are nonstructural. Noncapsid viral protein X (NCVPX, Mr ∿ 38,000), which is derived from the central portion of NCVP00, is associated with membranes of infected cells (Butterworth *et al.*, 1976), but its function has not yet been determined. Noncapsid viral protein 1b (NCVP1b, Mr ∿ 84,000) is derived from the 3' half of the genome. An analysis of recombinants between ts mutants suggested that this region coded for RNA replication functions (Cooper *et al.*, 1971). A number of noncapsid proteins

share tryptic peptides with NCVP1b. A protease found in EMC virus infected cells (p22, Mr ∿ 22,000) was shown by Palmenberg et al. (1980) to map in polypeptide D (analagous to poliovirus NCVP2). Noncapsid viral protein 2 (NCVP2, Mr ∿ 72,000) is derived from NCVP1b. In addition, preliminary tryptic mapping experiments (Palmenberg et al., 1980) and sequencing data (Kitamura et al., 1980) suggest that VPg (Mr ∿ 5,000-12,000), the genome-linked protein, maps in NCVP1b. Finally, a major viral polypeptide (p63 or NCVP4, Mr ∿ 58,000-63,000), which is associated with virus-specific RNA-dependent RNA polymerase activity, is also a subset of NCVP2 (Butterworth, 1973). The polymerase, the protease, and VPg appear to be distinct and probably do not share tryptic peptides with each other (Palmenberg et al., 1980). The possibility exists that several of the NCVP1b products, or NCVP1b itself, are involved in some phase of viral RNA replication. A number of other poliovirus-specific proteins are also present in infected cells. Many of them have been mapped, but their functions are not known.

Synthesis of Viral RNA

Poliovirus RNA replicates in the cytoplasm of the host cell and in close association with cellular membranes (Caliguiri and Tamm, 1970a and 1970b). Replication is independent of host macromolecular synthesis as it resists a number of inhibitors such as actinomycin D, α-amanitin, and cordycepin (Perez-Bercoff et al., 1974). During an infection the rate of virus-specific RNA synthesis follows a biphasic course. At early times (1-3 h) the amount of RNA synthesized increases exponentially and most of the newly synthesized

RNA molecules associate with ribosomes. Later (approximately 3-5 h) the rate of RNA synthesis is linear and results primarily in the production of virion RNA (Baltimore et al., 1966).

Studies on the species of viral RNA present in infected cells have contributed to a basic understanding of the mode of RNA replication. Three distinct species of RNA have been identified; they are (1) single-stranded 35S RNA that is free in the cytoplasm and associated with virions and polyribosomes, (2) double-stranded 20S RNA (replicative form or RF), and (3) replicative intermediate (RI) RNA which has both double-stranded and single-stranded character. When cumulative RNA synthesis is measured, replicative intermediate is a major species only during the exponential phase of RNA synthesis and the amount of RI present reaches a plateau at about 4 h. Viral ssRNA production is maximal during the linear phase and replicative form accumulates throughout the infection reaching its highest percentage at 6-8 h. At all times RI is the most rapidly labeled component (Noble and Levintow, 1970). In vitro experiments with membrane-bound replication complexes have demonstrated that RI is indeed an intermediate in the synthesis of viral ssRNA (Girard, 1969; McDonnell and Levintow, 1970). The role of RF in the replication cycle is still undetermined. Since RF accumulates in the infected cell with no apparent precursor relationship to virion RNA it has been considered a by-product of replication (Girard, 1969; McDonnell and Levintow, 1970). However, Perez-Bercoff (1979) has suggested that RF may also be a short-lived precursor to RI and that it accumulates late in the infectious cycle only because of the breakdown of the replication machinery. Either model is consistent

with current data, and the true function of RF in RNA replication remains to be determined.

The replicative intermediate is a complex structure consisting of one full length minus-strand RNA and approximately 6.5 nascent chains of plus-strand RNA. It is clearly the site for the synthesis of virion and messenger RNA. It is not known, however, whether the synthesis of minus strands takes place in a similar structure. Although minus strands comprise 5-10% of the RNA synthesized in an infection, the site and mechanism for their synthesis still remains to be determined. In addition, the mechanism for the asymmetric synthesis of plus strands is unknown.

An additional structural feature of poliovirus RNA which may have implications in its replication is the covalent linkage of the small protein, VPg, to the 5' end of the RNA molecule (Flanegan et al., 1977; Nomoto et al., 1977). The genome-linked protein is covalently bound to virion RNA by a phosphodiester linkage between the O^4 of the tyrosine residue in VPg and the 5'phosphate of the terminal uridylic acid residue in the RNA (Ambros and Baltimore, 1978; Rothberg et al., 1978). Moreover, it is linked to the 5'-terminus of the nascent plus strands of the RI, the 5'end of the plus strand of the RF, and the 5'-ends of the minus strands in the RF and the RI (Flanegan et al., 1977; Pettersson et al., 1978; Wu et al, 1978). Flanegan et al. (1977) and Nomoto et al. (1977) suggested that either VPg or VPg linked to one or more nucleotides might function as a primer for RNA replication. The presence of VPg on all newly synthesized RNAs is consistent with this proposal, but no data are yet available to substantiate the hypothesis.

Although the function of the poly(A) sequence at the 3' end of the virion RNA has not been determined, it may play a role in viral RNA synthesis. The 3' terminal poly(A) sequence in plus-strand RNA is apparently coded for by the poly(U) sequence that is present at the 5' end of the minus-strand RNA (Yogo and Wimmer, 1973 and 1975). Spector and Baltimore (1974) showed that poly(A)-deficient poliovirus RNA was not infectious in transfection experiments. Because it was still possible to efficiently translate the poly(A)-deficient RNA in *in vitro* translation experiments, it was proposed that the poly(A) sequence might be required for RNA replication. It is possible that the poly(A) sequence is important in the initial interaction of the replicase with the RNA template.

The Poliovirus Replicase

Studies on poliovirus RNA replication have, until recently, provided little information on the nature of the enzyme(s) required for this process. In 1963 an RNA-dependent RNA polymerase (replicase) was first demonstrated to be present in extracts of poliovirus infected cells (Baltimore *et al.*, 1963). Polymerase activity was shown by Eggers *et al.* (1963) to be dependent upon concurrent viral protein synthesis which suggests that the functional enzyme is short-lived. The appearance of the enzyme in infected cells was also shown to follow the same kinetics as viral RNA synthesis. This enzymatic activity was found in cytoplasmic extracts from infected cells but not uninfected cells and incorporated ribonucleotides into a heteropolymeric product. The polymerase activity measured in these experiments was not stimulated by the addition of an exogenous RNA template

and, therefore, was associated with endogenous enzyme-template complexes. The RNA synthesis associated with these complexes was apparently restricted to the completion of preexisting nascent strands (Girard, 1969).

Studies on the replication complex have been helpful in the discovery of the replicase and in establishing RNA precursor-product relationships. However, a detailed analysis of the enzymology of poliovirus RNA replication has awaited the development of an in vitro replication system which consists of purified components. The specific aims of this study were: (1) to construct such a system using a purified poliovirus-specific RNA polymerase that was dependent upon exogenous RNA for activity and that was able to utilize poliovirus RNA as a template; (2) to determine the polypeptides that were required for polymerase activity; and (3) to characterize the product synthesized in the in vitro reactions.

CHAPTER II
METHODOLOGY

Cell Culture

Cell Line

HeLa S3 cells were a gift of Drs. Janet and Gary Stern and Dr. Peter McGuire. They were grown in suspension culture in Joklik's minimum essential medium (MEM) supplemented with 7% calf serum. The cells were maintained at 3-6 x 10^5 cells/ml.

Virus Stocks

Poliovirus type 1 (Mohoney strain) was initially obtained from Dr. David Baltimore. Virus stocks containing about 10^{10} PFU/ml were prepared by infecting HeLa cell cultures with the virus for 6 h as described below. The infected cells were collected by centrifugation, resuspended in MEM at about 2 x 10^7 cells/ml and lysed by freezing and thawing. The nuclei and cellular debris were removed by centrifugation, and the stock virus solution was titered using monolayer cultures of HeLa cells. The virus stocks were stored at -20°C.

Preparation of Infected Cell Extracts

Suspension cultures of HeLa cells were infected with poliovirus type 1 as described by Villa-Komaroff *et al.* (1974). The cells (4 x 10^8) were collected by centrifugation and washed once with Earle's saline. The cell pellet was resuspended in the virus stock solution so that the multiplicity of infection was 20. After

allowing the virus to adsorb to the cells at ambient temperature for 30 min, Joklik's MEM, prewarmed to 37°C and containing 5% calf serum, was added to give a final concentration of 4×10^6 cells/ml. The infected culture was maintained with stirring at 37°C. At 15 min post-infection actinomycin D was added to a final concentration of 5 µg/ml. The cells were collected at 5 h post-infection, washed once in Earle's saline, and resuspended at 8×10^7 cells/ml in 10 mM Tris-hydrochloride, pH 8.0 - 10 mM NaCl (TN buffer). After swelling for 10 min, the cells were broken with 10 strokes of a Dounce homogenizer and the nuclei were removed by centrifugation at 900 x g for 5 min.

Radiolabeling of Infected Cells

Infected HeLa cells (100 ml at 4×10^6 cells/ml) were collected by centrifugation at 3 h post-infection, washed once in Earle's saline solution at 37°C and resuspended in 100 ml of Earle's saline supplemented with 3.5% dialysed calf serum, 3.5% dialysed fetal calf serum, one twentieth the standard concentration of Eagle's minimum essential medium amino acids (GIBCO), 2 mM glutamine, and 1 mCi [^{35}S]methionine. The cells were incubated at 37°C and were collected at 5 h post-infection as described above.

Enzyme Analysis

Enzyme Purification

A high speed supernatant of the cytoplasmic extract was prepared by centrifugation at 200,000 x g for 2 h at 4°C. The supernatant was placed on ice and solid ammonium sulfate (Schwarz/Mann) was gradually added with constant stirring until the solution was 35% saturated. The precipitate that formed was collected by

centrifugation at 10,000 x g for 10 min at 4°C. The pellet was resuspended in 1 ml of TN buffer-20% glycerol, diluted with 4 ml of 50 mM Tris(pH 8.0)-20% glycerol-0.1% Nonidet P40-2 mM dithiothreitol (DTT)-10 µg/ml ovalbumin (buffer A) and applied to a phosphocellulose column (0.8 by 6 cm) at a flow rate of 0.2 ml per min. The phosphocellulose had been prepared by the method of Burgess (1969), equilibrated with 50 mM Tris(pH 8.0) and then washed with 20 ml of buffer A. After washing the column with 50 ml of buffer A, the polymerase was eluted in 1 ml fractions with a 60-ml 0 to 1 M KCl gradient in buffer A. Peak fractions of polymerase activity were pooled, concentrated approximately fourfold against solid sucrose and layered onto a Sephacryl S-200 column (either 1.6 x 63 cm or 1.6 x 180 cm) that was previously equilibrated with buffer A-0.2 M KCl. The column was developed at a flow rate of 5 ml per h using buffer A-0.2 M KCl and by collecting 2 ml fractions. Peak fractions of activity were pooled and either dialysed against 50 mM Tris(pH 8.0)-50% glycerol-2 mM DTT-0.1 M KCl (buffer B) or loaded directly onto a 2 ml hydroxylapatite column. The column was washed with 10 ml of 0.05 M posassium phosphate (pH 7.5)-2 mM DTT-10% glycerol and the polymerase was then eluted in 1 ml fractions using 0.5 M potassium phosphate (pH 7.5)-2 mM DTT-10% glycerol. Peak fractions of polymerase activity were pooled, dialysed against buffer B and stored at -70°C. All operations were carried out at 0-4°C. Polymerase activity was assayed at each step using a poly(A) template and an oligo(U) primer as described below. All buffers were treated with 0.01% diethylpyrocarbonate (DEP) and autoclaved before use to remove nuclease contaminants.

Polymerase Assays

Polymerase activity was measured by using either poly(A) (Miles Laboratories) or purified poliovirion RNA as a template. When poly(A) served as the template the enzyme was assayed in a 60-µl solution containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 8.0), 3 mM $Mg(CH_3COO)_2$, 13 µM [5,6-^{3}H]UTP (8.6×10^3 cpm/pmol), poly(A) (2.5 µg), $oligo(U)_{20}$ (0.63 µg) and 10 mM DTT. Actinomycin D (10 µg/ml) was used in the assay for initial studies but was subsequently omitted. Reactions were carried at 30°C for 30 minutes.

The reaction conditions were modified slightly when poliovirion RNA was used as the template. In a standard reaction 2-5 µl of enzyme was assayed for 60 min at 30°C in a 30-µl solution containing: 42 mM HEPES buffer (pH 8.0), 3 mM $Mg(CH_3COO)_2$, 8 mM DTT, 45 µM [5,6-^{3}H]UTP (1×10^4 cpm/pmol), 0.8 mM each of ATP, CTP and GTP, 1-2 µg of poliovirion RNA and 0.02 µg of $oligo(U)_{20}$ per µg of virion RNA. The ratio of $oligo(U)_{20}$ to poliovirion RNA molecules in the assay varied with the lot of oligo(U) used. In all cases the oligo(U) to RNA ratio was optimized for incorporation under the standard reaction conditions. For the majority of assays oligo(U) was from Calbiochem and was treated with alkaline phosphatase prior to use. The ratio of oligo(U) to poliovirion RNA molecules was 8:1. In some experiments 10 µM [α^{32}P]UTP (33 Ci/mmol) or 0.8 µM [α^{32}P]GTP (410 Ci/mmol) was used as the labeled substrate. The other three triphosphates were present at a concentration of 0.8 mM.

When virion RNA was used in the polymerase assay, care was taken to use DEP-treated sterile tubes and pipette tips. In addition,

all solutions with the exception of the nucleotides were DEP-treated and autoclaved prior to use. The nucleotides were dissolved in 50 mM Tris-hydrochloride (pH 7.5) which had been treated with DEP and sterilized.

All reactions were stopped by adding 1 ml of cold 7% trichloroacetic acid (TCA), 2% sodium pyrophosphate and 100 µg of carrier yeast RNA. The labeled product was collected on membrane filters (Gelman GN-6, 0.45 µm) and counted in 5 ml Aquasol-2 scintillation fluid (New England Nuclear Corp.). The background generated from reactions without added enzyme was subtracted from each measurement. One unit of activity is equivalent to the amount of enzyme required to incorporate 1 nmol of labeled substrate into acid-insoluble product in 30 min at 30°C when poly(A) serves as a template.

Protein Determination

Protein concentrations were measured by the method of Bradford (1976). Protein was detected by adding 1 ml Coomassie reagent [0.01% Coomassie blue G-250 in 4.7% ethanol, 8.5% (W/V) phosphoric acid] to 0.1 ml of solution containing 1-10 µg of protein. The optical density at 595 nm (OD_{595}) was measured and values were compared to those obtained using the bovine serum albumin (BSA) standard.

Glycerol Gradient Centrifugation

Sedimentation values of the polymerase were determined by centrifugation in 15-30% glycerol gradients. Generally, 0.5 ml of enzyme solution was layered onto a 10.6 ml 15-30% glycerol gradient in TN buffer at 4°C. The samples were centrifuged at 40,000 rpm for 28 h at 4°C in a Beckman SW41 rotor. Gradients were collected in 0.5 ml fractions on ice by pumping from the bottom of the tube.

Fractions were then assayed for activity with poly(A)·oligo(U). Bovine serum albumin (0.5-1 mg) was centrifuged in a parallel tube as a sedimentation marker. The BSA peak was located by measuring the OD_{280} of each fraction.

Nucleic Acid Preparation

Purification of Poliovirion RNA

Infected cells were collected a 6 h post-infection, washed once in Earle's saline and lysed by resuspending the cells in cold 1% Nonidet P-40, 10 mM Tris-hydrochloride (pH 8.0), 10 mM NaCl. The nuclei were removed from the extract by centrifugation at 900 x g for 5 min and SDS was then added to give a final concentration of 0.5%. The virions were collected by centrifugation at 45,000 rpm for 2 h in a Beckman 50.1 rotor and were resuspended in 10 mM Tris-hydrochloride (pH 7.5), 0.1 M NaCl, 0.001 M EDTA, 0.5% SDS (buffer C). The virus was further purified by sedimentation in a sucrose gradient or by banding in a cesium chloride (CsCl) density gradient. When purified in a sucrose gradient, the virus solution was layered on a 15-30% gradient in buffer C and centrifuged at 24,000 rpm for 3 h at 22°C in a Beckman SW27 rotor. The gradient was collected in 1 ml fractions by pumping from the bottom of the tube, and the virus was detected by UV absorption at 260 nm. When banded in cesium chloride, the virus was added to a solution of CsCl in buffer C containing 1% Brij-35. The final volume of the solution was 9 ml and its density was approximately 1.34 g/cc. The mixture was centrifuged at 40,000 rpm in a Beckman Ti50 rotor for 18 h at 22°C. The visible band of poliovirus was collected and diluted with buffer C. The virus was

collected by centrifugation in a Beckman Ti50.2 rotor and was resuspended in 1 ml of buffer C. Poliovirus RNA was released from the virions by the addition of 0.1 ml 10% SDS and 0.3 ml 0.1 M sodium acetate (pH 3.5) and was purified by sedimentation in a 15-30% sucrose gradient in buffer C. The gradients were centrifuged at 22,000 rpm in a Beckman SW27 rotor for 22.5 h at 22°C. The gradients were collected in 1 ml fractions by pumping from the bottom, and the 35S poliovirion RNA peak was located by measuring the absorbance at 260 nm of each fraction. HeLa ribosomal RNAs served as markers in a parallel gradient. The poliovirion RNA was precipitated in 70% ethanol and was stored at -20°C. Shortly before use the RNA was collected by centrifugation and was resuspended in 0.1 mM EDTA (pH 7.0). The EDTA solution was treated with 0.01% DEP and autoclaved before use to inactivate nuclease contaminants. The purified RNA was shown to be intact by its electrophoretic mobility in 1% agarose gels relative to 18S and 28S HeLa ribosomal RNA markers.

Preparation of Poliovirus Double-Stranded RNA

A cytoplasmic extract of infected cells was prepared as described above. Sodium dodecyl sulfate was added to the solution to a final concentration of 1% and proteins were removed from the sample by phenol extraction. Phenol (0.5 volume, redistilled and saturated with H_2O) and 0.5 volume chloroform (24):isoamyl alcohol (1) were added to the sample, mixed well and centrifuged at 10,000 x g for 10 min. The phenol phase was removed, and the above extraction was repeated twice again. Phenol was removed from the aqueous phase by three extractions with an equal volume of chloroform:isoamyl alcohol.

The RNA was precipitated by adding 2.5 volumes of ethanol and was resuspended in buffer C. Lithium chloride was added to the solution to a final concentration of 2 M. The sample was stored at 0°C overnight and then centrifuged at 10,000 x g for 10 min to remove single-stranded RNA. The double-stranded RNA was precipitated from the supernatant with ethanol, resuspended in 1.5 ml of buffer C and then centrifuged through a 15-30% sucrose gradient as described above. The double-stranded RNA in the 20S region of the gradient was precipitated with ethanol and was stored as a suspension at -20°C.

Alkaline Phosphatase Treatment of Oligo(U)

To achieve maximal polymerase activity some lots of oligo(U) were treated with bacterial alkaline phosphatase prior to use in the assay. The oligo(U) (10 mg/ml in 10 mM Tris-hydrochloride, pH 8.0, 120 mM NaCl) was treated with 1 unit of bacterial alkaline phosphatase (Bethesda Research Labs) for 45 min at 37°C. The enzyme was removed by phenol extraction as described above. The oligo(U) was precipitated from the aqueous phase by the addition of 2.5 volumes 95% ethanol and 0.1 volume 4 M sodium acetate. The precipitate was collected by centrifugation, resuspended in sterile H_2O to a final concentration of 4 mg/ml and stored at -20°C.

Preparation of Labeled Oligo(U)

Oligo(U) was labeled at the 5' end using [γ^{32}P]ATP and polynucleotide kinase. The reaction contained 70 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 1 µM [γ^{32}P]ATP (3125 Ci/mmol), 10 µg oligo(U) and 10 units of T4 polynucleotide kinase (Bethesda Research Labs) and was incubated at 37°C for 30 min. The labeled oligo(U) was hybridized to a 1 ml poly(A) sepharose

column (P-L Biochemicals) which had been equilibrated with 10 mM Tris-hydrochloride (pH 7.5), 1.0 mM EDTA, 0.5 M NaCl, 0.05% SDS (binding buffer). The column was washed extensively with binding buffer and the oligo(U) was eluted with 10 mM Tris-hydrochloride (pH 7.5), 1.0 mM EDTA, 0.05% SDS. Fractions containing ^{32}P were pooled and the sample was extracted with phenol/chloroform/isoamyl-alcohol as described above. The oligo(U) was precipitated in 70% ethanol, collected by centrifugation and resuspended in sterile 0.1 mM EDTA. When further purified by high voltage electrophoresis, the oligo(U) was applied to 3MM paper (Whatman) and electrophoresed at pH 3.5 for 2 h at 30 V/cm. It was located by autoradiography, eluted from the paper with H_2O, lyophylized and resuspended in sterile 0.1 mM EDTA.

Gel Electrophoresis

Polyacrylamide Gel Electrophoresis

Proteins were analyzed in SDS-polyacrylamide gels by the method of Laemmli (1970) except that the running gel buffer was 0.19 M Tris-hydrochloride (pH 8.0), the stacking buffer was 0.06 M Tris-hydrochloride (pH 6.8) and the electrode buffer was 0.05 M Tris base, 0.4 M glycine, 0.1% SDS. Proteins were precipitated with 20 µg carrier cytochrome c and 6-10 volumes of acetone. The pellet was resuspended in 30 µl of sample buffer (0.065 M Tris-hydrochloride, pH 6.8, 2% SDS, 20% glycerol, 0.02 M β-mercaptoethanol) and the sample was layered onto a discontinuous 10% polyacrylamide gel crosslinked with 0.27% bis acrylamide and containing 0.1% SDS. Electrophoresis was carried out at 150 V (constant voltage) for 3 h.

The gels were stained with Coomassie brilliant blue R250 (0.25% in 40% ethanol, 7% acetic acid) dried and autoradiographed at -70°C using Dupont Cronex x-ray film. To increase the sensitivity of detection, the fluorography procedure described by Bonner and Laskey (1974) was used for most gels. The molecular weight markers used to calibrate the gels were phosphorylase b (94,000d), catalase (60,000d), bovine serum albumin (67,000d), and ovalbumin (46,000d).

Iodination of Molecular Weight Markers

Protein samples were iodinated by the method of Bolton and Hunter (1973). The ^{125}I-labeled Bolton-Hunter reagent (4000 Ci/mmol), was dried with a stream of N_2 before use and resuspended in the original volume with 0.1 M sodium borate buffer, pH 8.5. The molecular weight markers (5 µg total), were precipitated with ten volumes of acetone. Pellets were dried *in vacuo*, resuspended in 10 µl Bolton-Hunter reagent (100 µCi), and incubated on ice for 1.5 h. One volume of 1 M Tris-hydrochloride, pH 7.5 was added and the proteins were precipitated twice with 10 volumes of acetone and 20 µg carrier cytochrome c.

Two Dimensional Analysis of Proteins

The ^{35}S-labeled viral proteins were analyzed by isoelectric focusing in the first dimension and by SDS-polyacrylamide gel electrophoresis in the second dimension using methods similar to those described by O'Farrell (1975) and by Horst and Roberts (1979). The labeled viral proteins were first treated with ribonuclease A (15 µg) for 1 h at 25°C to remove any residual RNA. The samples were then made 9.5 M in urea, 2% in carrier ampholytes (1.2% pH range 5-7 and 0.8% pH range 3-10) and 5% in β-mercaptoethanol. In the

first dimension, the samples were subjected to isoelectric focusing after layering onto a 4.3% polyacrylamide gel crosslinked with N,-N'-diallyltartardiamide (Bio-Rad). The gels contained 9 M urea, 2% NP40, 1.2% carrier ampholytes pH 5-7, and 0.8% carrier ampholytes pH 3-10. The upper chamber was filled with degassed 0.04 M NaOH and the lower chamber with 0.06 M H_2SO_4. The gels were run at 75 volts for 30 minutes, 150 volts for 1 h, 300 volts for 15 h, and 450 volts for 1 h. The temperature was maintained at 18°C. After focusing, the gels were equilibrated in 0.06 M Tris(pH 6.8)-1% SDS-1% β-mercaptoethanol containing bromphenol blue for 1-2 h. For the second dimension, the tube gels were fixed in position above a 10% polyacrylamide slab gel using 1% agarose in equilibration buffer and were subjected to electrophoresis as described above. Molecular weights were determined by the electrophoresis of a reference sample in the same gel. The pH gradient formed in the first dimension was determined by measuring the pH of 5 mm gel slices in 0.4 ml of degassed 9.2 M urea.

Methylmercury Agarose Gel Electrophoresis

Product RNA which was labeled with [α^{32}P]NTP was analyzed by the method of Bailey and Davidson (1976) on agarose gels containing the denaturing agent methylmercury hydroxide (CH_3HgOH). Product was synthesized in the presence of [α^{32}P]GTP in a standard reaction. The RNA was precipitated from the reaction with 70% ethanol, collected by centrifugation and resuspended in 10 µl of 1.5 x buffer (1 x buffer = 0.05 M boric acid, 0.005 M $Na_2B_4O_7 \cdot 10\ H_2O$, 0.01 M sodium sulfate, 0.001 M EDTA, pH 8.2). Methylmercury was added to a final concentration of 15 mM and the sample was incubated at

room temperature for 10 min. An equal volume of a solution containing 15 mM CH_3HgOH, 0.1% bromphenol blue and 50% glycerol was added and the samples were layered on a 1% vertical agarose gel in 1 x buffer containing 5 mM CH_3HgOH. Electrophoresis was carried out at 110 V for 3 h. Gels were then soaked for 30 min in 0.5 M $NH_4C_2H_3O_2$ containing 0.5 µg/ml ethidium bromide, photographed using Polaroid T57 film, dried and autoradiographed. All gels were prepared and run in a chemical hood and all materials contaminated with CH_3HgOH were soaked in 0.5 M $NH_4C_2H_3O_2$, 0.01 M β-mercaptoethanol.

Analysis of Product RNA

Hybridization Analysis

Product RNA labeled with [α^{32}P]NTP was precipitated from a standard reaction with cold 5% TCA. The precipitate was collected by centrifugation, suspended in 0.1 M HEPES (pH 8.0), 0.4 M sodium acetate and reprecipitated with 2.5 volumes of ethanol. The RNA pellet was dissolved in 0.01 M Tris (pH 7.5) and the sample was divided into five aliquots, each containing 14,000 cpm of product with a final volume of 0.4 ml. Ten micrograms of poliovirion RNA or 10 µg of HeLa RNA were added to some tubes. Samples were boiled for 10 min and then annealed for 60 min at 50°C after the addition of NaCl to a final concentration of 0.2 M. The samples were chilled and divided into two equal portions, one of which was treated with RNases T1, T2 and A (0.02, 0.005, 0.15 units per ml, respectively) for 30 min at 20°C. All samples were then precipitated with 7% TCA and the RNase resistant RNA was collected on a membrane filter and counted.

Nearest Neighbor Analysis

Product RNA was synthesized in the presence of either [α^{32}P]GTP or [α^{32}P]UTP under standard reaction conditions. Labeled product was precipitated with 1.0 ml cold 5% TCA, collected by centrifugation, resuspended in 0.05 M Tris(pH 8.0), 0.1 mM EDTA, 0.4 M sodium acetate and reprecipitated with 70% ethanol. When the RNA was digested with alkali, the precipitate was resuspended in 0.5 M KOH and incubated at ambient temperature for 20 h. The solution was neutralized with dilute perchloric acid, the $KClO_4$ precipitate was removed by centrifugation at 4°C and the sample was lyophylized. When digested with RNase the sample was resuspended in 10 mM Tris(pH 8.0) containing 200, 5 and 150 units per ml of RNases T1, T2 and A, respectively. After heating at 100°C for 10 min, more RNase was added to the sample and it was incubated for 1 h at 37°C. All samples were analysed by high voltage electrophoresis. Samples of 20 µl were spotted in 1 µl aliquots onto Whatman 3MM paper. The paper was wetted with 0.3% pyridine, 3% acetic acid (pH 3.5) and the samples were electrophoresed in the same buffer at 30 V/cm until the xylene cyanol dye was 10 cm from the origin. The paper was dried and autoradiographed to locate the 3'-mononucleotides. Each spot was then cut from the paper and counted in 5 ml Aquasol-2 scintillation fluid.

Materials

The [5,6-^{3}H]UTP was obtained from Schwarz/Mann in a 50% ethanol solution. The ethanol was removed and the solution volume was reduced fivefold. The [^{35}S]methionine, [γ^{32}P]ATP and the

Bolton-Hunter reagent were from New England Nuclear Corporation. The [α^{32}P]GTP and [α^{32}P]UTP were from Amersham. Oligo(U), the 5'nucleoside triphosphates and RNases T1 and T2 were obtained from Calbiochem. Ribonuclease A was from Worthington. Carrier ampholytes were purchased from Bio-Rad Laboratories. Poly(U) Sepharose 4B and Sephacryl S-200 were from Pharmacia. Phosphorylase b, catalase, bovine serum albumin, ovalbumin and cytochrome c were from Sigma. Methylmercury hydroxide was obtained from Alfa Products and HGT agarose was from Miles Laboratories. Actinomycin D was a gift from Merck, Sharp, and Dome.

CHAPTER III
PURIFICATION OF THE POLIOVIRUS RNA-DEPENDENT RNA POLYMERASE AND CHARACTERIZATION OF ITS *IN VITRO* ACTIVITY

Introduction

Early attempts to purify the poliovirus RNA-dependent RNA polymerase were limited to the purification of the replicase complexed to its endogenous RNA template (Girard *et al.*, 1967; Ehrenfeld *et al.*, 1970; Lundquist *et al.*, 1974; Röder and Koschel, 1974; Butterworth *et al.*, 1976; Yin, 1977). The endogenous replication complex was not stimulated by the addition of an exogenous RNA template and was not able to initiate the synthesis of new RNA chains. The activity associated with the complex resulted from the elongation of nascent RNA chains that were present in the complex at the time it was isolated. Attempts to isolate an active polymerase from the replication complex has not been very successful. This is probably due to the harsh detergent treatment that was required to separate the polymerase from its endogenous RNA template. Traub *et al.* (1976) reported the partial purification of an RNA-dependent RNA polymerase activity which was eluted from the replication complex isolated from EMCV-infected cells. The activity was dependent upon an added RNA template but was very unstable.

A template-dependent RNA polymerase was isolated from a solublized membrane fraction of poliovirus-infected cells by

Flanegan and Baltimore (1977). This enzyme copied a poly(A) template in the presence of an oligo(U) primer and sedimented at about 4 S on a glycerol gradient. The poly(U) polymerase was not present in uninfected cells, appeared in the extracts of infected cells at about 2 h post-infection and increased in activity until about 5 h post-infection. Another poly(U) polymerase with similar properties was also recently isolated from the membranes of FMDV-infected cells (Polatnick, 1980).

An enzyme similar to the membrane-bound poly(U) polymerase was detected in the soluble phase of a poliovirus infected cell extract (Flanegan and Baltimore, 1979). This enzyme was partially purified from a 200,000 x g supernatant of the cytoplasmic extract by glycerol gradient centrifugation and phosphocellulose chromatography. The activity of the soluble polymerase also depended upon the presence of both poly(A) and oligo(U).

In this study we have purified the soluble poliovirus polymerase by ammonium sulfate precipitation, phosphocellulose chromatography, gel filtration in Sephacryl S-200, and hydroxylapatite chromatography. The purified enzyme was shown to copy a poliovirion RNA template in the presence of an oligo(U) primer and the four ribonucleoside triphosphates. Several additional parameters of the <u>in vitro</u> polymerase reaction were also characterized in this study.

Results

Polymerase Purification

The poliovirus RNA polymerase was purified as a soluble protein from HeLa cell extracts at 5 h post-infection. A mixture of the homopolymers poly(A) and oligo(U) were used to assay for polymerase activity during the purification.

More than 90% of the endogenous RNA polymerase activity was removed from the infected cell extracts by centrifugation at 200,000 x g for 2 h (data not shown). The poly(U) polymerase activity in the supernatant (S200) represented about one third of the activity that was present in the original extract (Flanegan and Van Dyke, 1979).

Ammonium sulfate precipitation was used to concentrate and purify the polymerase that was present in the S200 (Table 1, fraction II). The majority of polymerase activity precipitated when the S200 was about 30% saturated with ammonium sulfate (Fig. 1A). For this reason, 35% ammonium sulfate was routinely used to precipitate the polymerase at this step in the purification. This yielded about an elevenfold purification of the enzyme (Table 1). The recovery at this step was about 250% (Table 1), suggesting that an inhibitor of polymerase activity was present in the S200.

In our initial attempts to purify the polymerase, the fraction II enzyme was sedimented in a glycerol gradient. At this stage in purification the enzyme sedimented at about 7 S. This procedure, however, resulted in about a 30% loss of activity and did not increase the specific activity of the polymerase (Flanegan and

Van Dyke, 1979). This step was therefore omitted, and the fraction II enzyme was subsequently adsorbed to a phosphocellulose column. The polymerase eluted from phosphocellulose in a single peak when the KCl concentration in the elution gradient was about 0.15 M (Fig. 1B). This step resulted in about a sevenfold purification of the enzyme (Table 1, fraction III).

The fraction III enzyme was gel filtered on a Sephacryl S-200 column (1.6 x 180 cm). The poly(U) polymerase eluted as a single peak between the protein markers ovalbumin and cytochrome c in a position corresponding to a molecular weight of 20,000d (Fig. 1C). When the KCl concentration of the buffer used to develop the Sephacryl column was decreased from 0.2 M to 0.05 M, the polymerase eluted after the cytochrome c marker (data not shown). Increasing the KCl concentration to 0.5 M had no effect on the position of elution, but resulted in an eightfold decrease in the yield of enzyme (Table 1, fraction IV). To maximize the yield of fraction IV enzyme in subsequent preparations, a shorter Sephacryl column (63 cm) was used and was developed in 0.2 M KCl. The enzyme that eluted from the Sephacryl column (fraction IV) was relatively unstable, possibly due to the low concentration of protein.

The fraction IV enzyme was concentrated and further purified by chromatography on a hydroxylapatite column. Preliminary experiments showed that the polymerase eluted from hydroxylapatite at a phosphate concentration between 0.25 M and 0.5 M (data not shown). Therefore, 0.5 M potassium phosphate, pH 7.5 was normally used to batch-elute the enzyme from hydroxylapatite. Further concentration of the enzyme was achieved by dialysis against 50 mM Tris-hydrochloride

TABLE 1. Purification of poliovirus RNA polymerase.

Purification Step[a]		Volume (ml)	Total Units[b]	Protein (mg/ml)	Sp. Act. (U/mg)[b]	Yield (%)
I.	Supernatant (200,000 x g)	5.6	14.4	2.3	1.1	100
II.	Ammonium Sulfate	1.0	35.3	2.9	12.2	246
III.	Phosphocellulose	1.15	24.1	0.25	83.4	168
IV.	Sephacryl S-200 (1.6 x 180 cm)	28.0	0.54	ND[d]	ND	3.8[c]
V.	Hydroxylapatite	0.9	0.34	ND	ND	2.3

[a]Purification represented here is the same that is analyzed in Fig. 6.

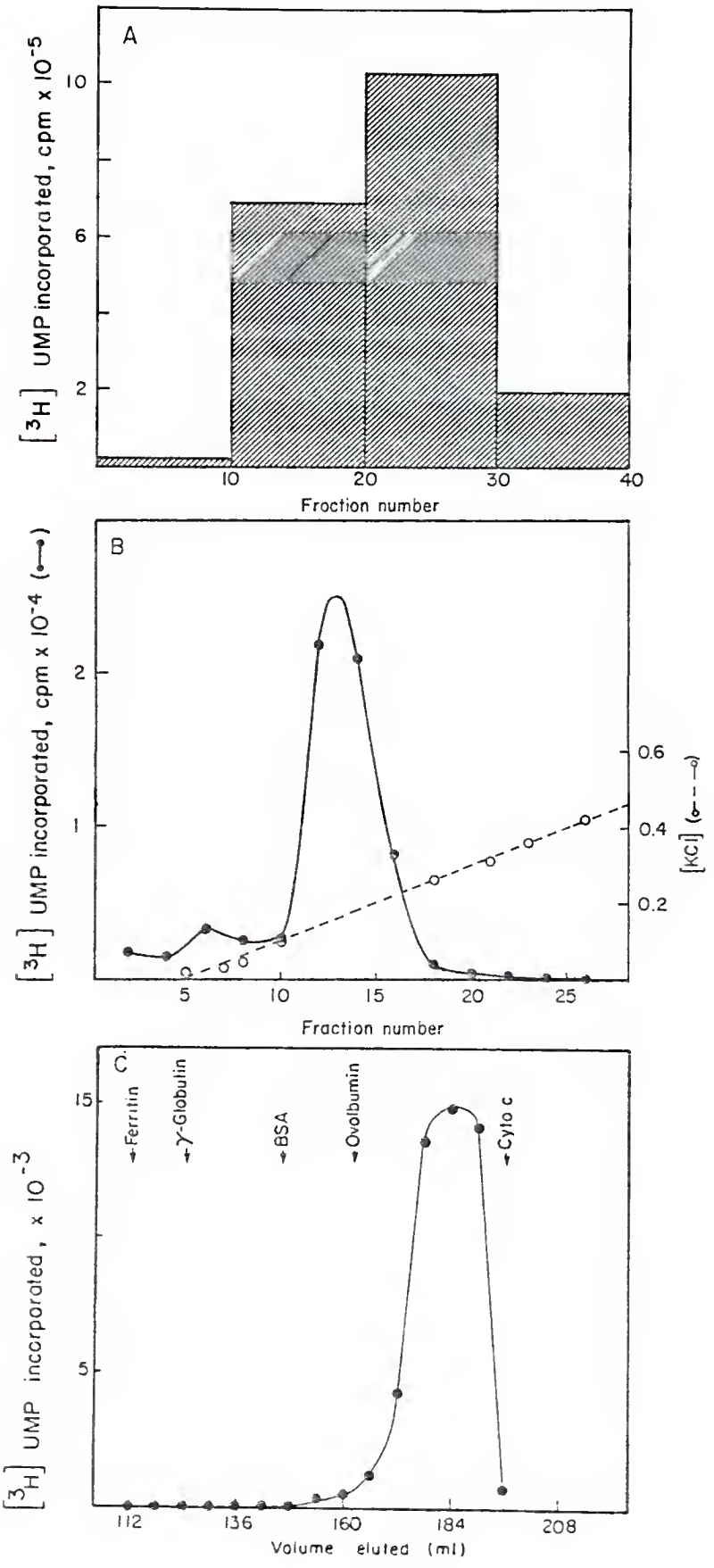
[b]One unit of activity is equivalent to the amount of enzyme required to incorporate 1 nmol of labeled substrate into acid-insoluble product in 30 min at 30°C under the reaction conditions specified in the text. Poly(A) and oligo(U) were used to measure polymerase activity at each stage of purification.

[c]In this experiment, the yield from the Sephacryl column was significantly lower than usual. The yield of input activity in two other experiments was 40 and 31%. The Sephacryl column used in this experiment was developed in 0.5 M KCl instead of 0.2 M KCl. KCl is known to inhibit the activity of the enzyme and may also affect its stability.

[d]ND, Not determined.

Figure 1.

Purification of the poliovirus RNA polymerase. (A) Recovery of polymerase activity after ammonium sulfate precipitation. A high-speed supernatant was prepared from infected cells at 5 h post-infection and solid ammonium sulfate was added stepwise. The precipitates formed were collected by centrifugation, resuspended and assayed for activity. (B) Chromatography of the polymerase on phosphocellulose. The polymerase that was recovered after ammonium sulfate precipitation was chromatographed on a phosphocellulose column as described in Chapter II. Conductivity measurements with a Radiometer conductivity meter were used to determine the salt concentration in the elution gradient. (C) Gel filtration of the polymerase on Sephacryl S-200. The peak fractions from the phospho-cellulose column were pooled, concentrated, and chromatographed on a Sephacryl S-200 column (1.6 x 180 cm). The column was calibrated with the protein standards ferritin, human γ-globulin, bovine serum albumin (BSA), ovalbumin and cytochrome c (cyt c). A poly(A) template and an oligo(U) primer were used to measure polymerase activity in all of the above procedures.


A
[3H] UMP incorporated, cpm x 10-5
10
6
2
10
20
30
40
Fraction number
B
[3H] UMP incorporated, cpm x 10-4 (•—•)
[KCl] (o- - -o)
2
1
0.6
0.4
0.2
5
10
15
20
25
Fraction number
C
[3H] UMP incorporated, x 10-3
15
5
Ferritin
γ-Globulin
BSA
Ovalbumin
Cyto c
112
136
160
184
208
Volume eluted (ml)

(pH 8.0), 2 mM DTT, 0.1 M KCl, 50% glycerol. Sixty percent of the activity present in the fraction IV polymerase was recovered after hydroxylapatite chromatography and dialysis (Table 1, fraction V). Fraction V polymerase was free of detectable ribonuclease (see Chapter V, Fig. 1B) and could be stored for up to six months at -70°C with little loss in activity.

Polymerase Activity on Poly(A)

Poly(U) polymerase activity was previously shown to be totally dependent on a poly(A) template, an olgio(U) primer and Mg^{2+}. The product of the reaction was sensitive to RNase and alkali and was resistant to DNase (Flanegan and Baltimore, 1977). The amount of product synthesized in a 30-min reaction by a partially purified preparation of polymerase (Table 1, fraction III) was found to be a linear function of enzyme concentration (Fig. 2A). Therefore, 2 µg or less of enzyme was typically used in the reactions. The optimum temperature for the incorporation of labeled substrate into RNA product in a 30-min reaction was 30°C (Fig. 2B). The rate of the poly(U) polymerase reaction at 30°C remained constant for at least 1 h (data not shown).

Polymerase Activity on Poliovirus RNA

The purified polymerase (Table 1, fraction V) was tested for activity on poliovirion RNA. In the presence of poliovirion RNA, oligo(U), and all four ribonucleoside triphosphates the enzyme incorporated [^{3}H]UMP into an acid insoluble product at a rate which was linear for at least 1 h (Fig. 3). Omission of oligo(U) or poliovirion RNA from the reaction resulted in a total loss of activity (Fig. 3 and Table 2). Deleting ATP, CTP, and GTP from a reaction

Figure 2.

Polymerase activity as a function of enzyme concentration and temperature. (A) The polymerase was purified as described in Table 1, and the amount of [^{3}H]UMP incorporated was measured as a function of the amount of fraction III polymerase used in the assay. (B) The amount of labeled substrate incorporated in a 30-min assay was measured as a function of the assay temperature. A poly(A)-oligo(U) template primer was used to measure polymerase activity in these experiments.

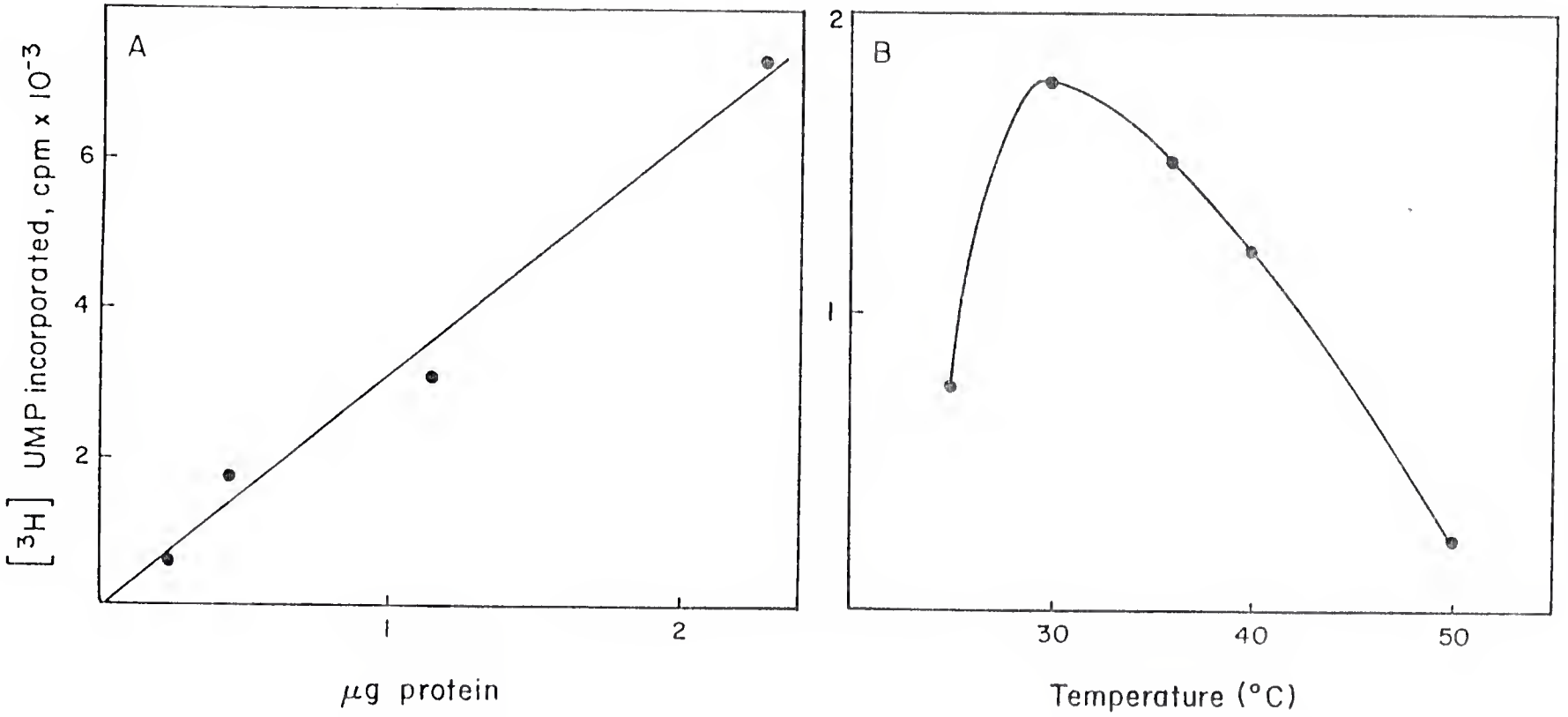
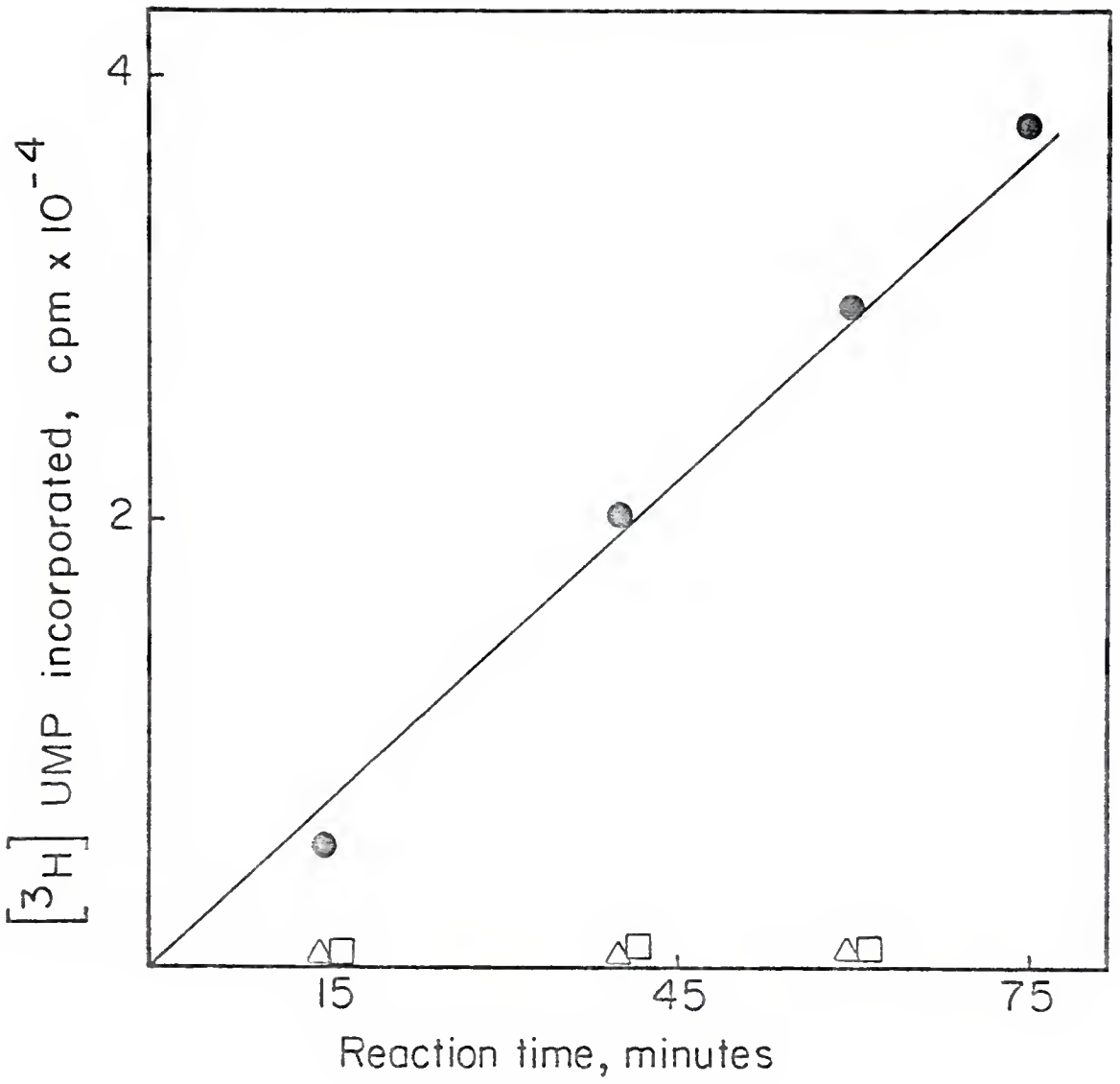

A
B
[3H] UMP incorporated, cpm x 10-3
6
4
2
1
2
1
2
30
40
50
μg protein
Temperature (°C)

Figure 3.

Activity of poliovirus RNA polymerase on a poliovirion RNA template. The polymerase (Table 1, fraction V) was assayed for activity by measuring the incorporation of [^{3}H]UMP into an acid-insoluble product. Incorporation was measured with poliovirion RNA, oligo(U), and all four ribonucleoside triphosphates (o); poliovirion RNA and all four ribonucleoside triphosphates (Δ); and oligo(U) and all four ribonucleoside triphosphates ( ).


[3H] UMP incorporated, cpm x 10-4
4
2
15
45
75
Reaction time, minutes

that contained [^{3}H]UTP as the labeled substrate resulted in a 90% reduction in the incorporation of labeled substrate (Table 2). This activity probably results from the synthesis of poly(U) on the poly(A) sequence at the 3'end of poliovirion RNA. In other experiments in which [^{3}H]GTP was used as the labeled substrate, omission of ATP, CTP, and UTP from the reaction resulted in a complete loss of activity (Flanegan and Van Dyke, 1979).

Polymerase activity on poliovirion RNA required the presence of Mg^{2+} (Table 2). The incorporation of [^{3}H]UMP into acid insoluble product was optimal when the Mg^{2+} concentration was about 3 mM (Fig. 4A). The presence of 3 mM EDTA in the reaction resulted in a tenfold reduction in polymerase activity (Table 2). Low concentrations of Mn^{2+} ($\sim$0.1 mM) could replace Mg^{2+} in the reaction, whereas higher concentrations were inhibitory (data not shown). When Mg^{2+} was present in the reaction, the addition of 0.05 mM Zn^{2+} or 0.1 mM Mn^{2+} stimulated polymerase activity by 10-40% (data not shown). Higher concentrations of Zn^{2+} (3 mM) and Mn^{2+} (1 mM) inhibited the activity of the polymerase by 100% (data not shown) and 34% (Table 2), respectively.

Addition of the polyanion heparin (12 µg) to the polymerase reaction resulted in a greater than 99% reduction in incorporation (Table 2). Any increase in the KCl concentration of the reaction was also inhibitory to polymerase activity (Fig. 4B). The degree of inhibition at any KCl concentration was about the same for both poly(A) and poliovirus RNA (Fig. 4B).

The polymerase was maximally active on poliovirion RNA when HEPES was used as the buffer in the standard reaction. Although

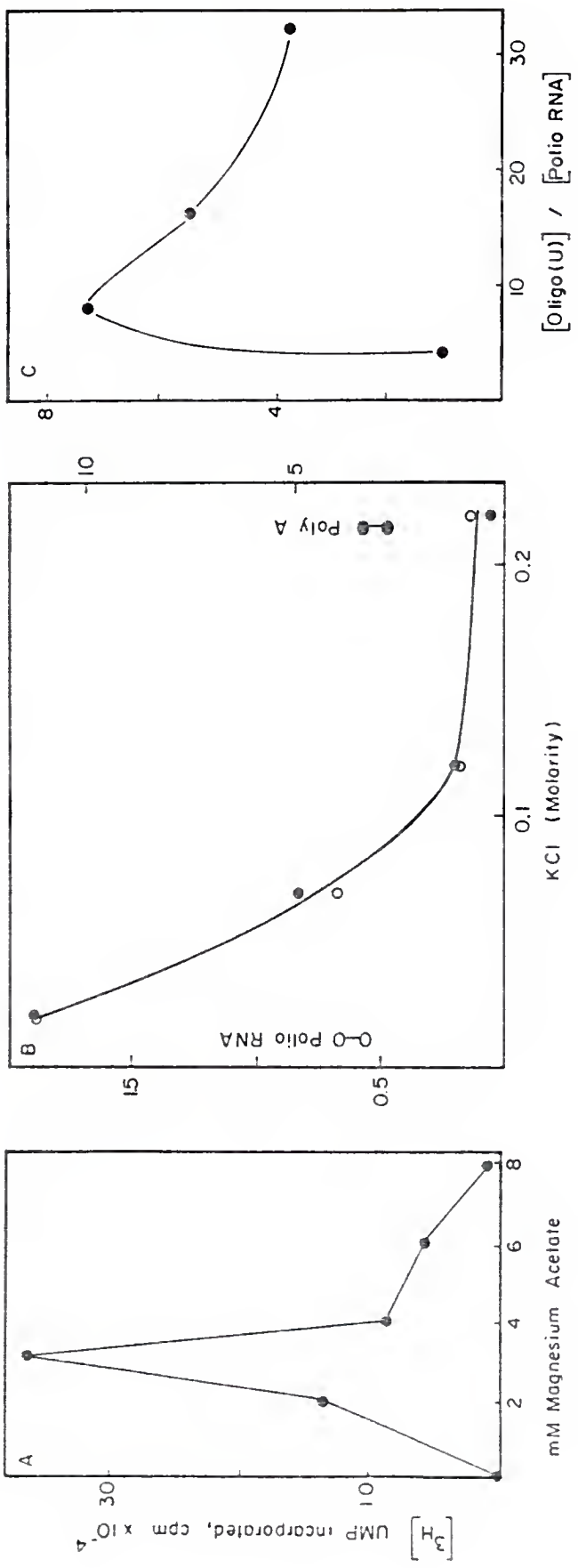
TABLE 2. Properties of the *in vitro* poliovirus RNA polymerase reaction.

Reaction components[a]	% optimal activity
I. Poliovirion RNA assay	
Complete system	100
Minus poliovirion RNA	0
Minus ATP, CTP, GTP	7
Minus oligo(U)	1
Minus Mg^{2+}	1
Minus DTT	90
Plus 3 mM EDTA	9
Plus 1 mM Mn^{2+}	34
II. Poly(A) assay	
Complete system	100
Plus 12 µg heparin	<0.03

[a]Reactions were for 60 min under standard reaction conditions using poliovirion RNA·oligo(U). As indicated above, various components were left out of the reaction or added to the reaction mixture.

Figure 4.

Effect of Mg^{2+}, KCl, and oligo(U) concentrations on polymerase activity. (A) Effect of Mg^{2+} concentration. The activity of the polymerase (Table 1, fraction III) was measured on virion RNA·oligo(U) at various $Mg(CH_3COO)_2$ concentrations. Other reaction conditions were as described in Chapter II. (B) Effect of increasing KCl concentration. The activity of the fraction III polymerase (Table 1) was measured on poly(A)·oligo(U) and virion RNA·oligo(U) after adding increasing amounts of KCl to the reaction mixtures. The KCl concentrations include the KCl present in the fraction III polymerase. The reactions were carried out under the standard conditions used for the poliovirus RNA assay (Chapter II). (C) Effect of changing the oligo(U)·virion RNA ratio. The activity of the polymerase was measured with a fixed amount of poliovirion RNA (1.5 µg) and with increasing amounts of oligo(U) (0.015 to 0.12 µg). The ratio of the number of oligo(U) molecules to the number of virion RNA molecules was calculated assuming a chain length of 20 nucleotides for oligo(U) and 7,500 nucleotides for poliovirus RNA (Granboulan and Girard, 1969). The standard reaction conditions were used and the assays were for 60 min at 30°C, using 2 µl of fraction V polymerase.


A
[3H] UMP incorporated, cpm x 10-4
mM Magnesium Acetate
B
Polio RNA
Poly A
KCl (Molarity)
C
[Oligo(U)] / [Polio RNA]

incorporation was maximum using HEPES at pH 7 the activity was only reduced by about 10% when the pH was increased to 8 (Table 3). The difference in the amount of incorporation that resulted when the polymerase was assayed at pH 7 and pH 8 varied and depended upon the time of reaction (data not shown). When the polymerase was assayed in sodium acetate, pH 4-5; Tris-hydrochloride, pH 7.5-8.0; PIPES, pH 6.0-7.5; sodium phosphate, pH 7-8; or sodium borate, pH 9.0-9.5, the incorporation was low and ranged from 2 to 21% of the control (Table 3). Incorporation was about 50% of the optimal value when TES at pH 8.0 was used and ranged from 50 to 92% of the control when sodium borate at pH 8.5 was used in the reaction (Table 3 and unpublished results). For most of the polymerase reactions described in this study HEPES at pH 8.0 was used as the buffer.

The temperature of the reaction affected the amount of labeled substrate incorporated into product RNA and the length of time that the incorporation rate remained linear. When assayed at 30°C, the in vitro reaction was linear for about 70 min (Fig. 3). Increasing the temperature to 37°C, however, resulted in a decrease in the rate of incorporation after 15 to 30 min (data not shown). The effect of temperature on the reaction rate was therefore determined by measuring the incorporation rates before 20 min at 25.5°, 30°, 34° and 38°C (Fig. 5). The rate of incorporation was lowest when the reaction temperature was 25.5°C (slope = 116 cpm/min). The rate increased 3.6-fold when the temperature was raised to 30°C (slope = 420 cpm/min) and another 2-fold when the temperature was increased to 34°C (slope = 840 cpm/min). Increasing the temperature to 38°C did

TABLE 3. Effect of pH and buffer on polymerase activity *in vitro*.

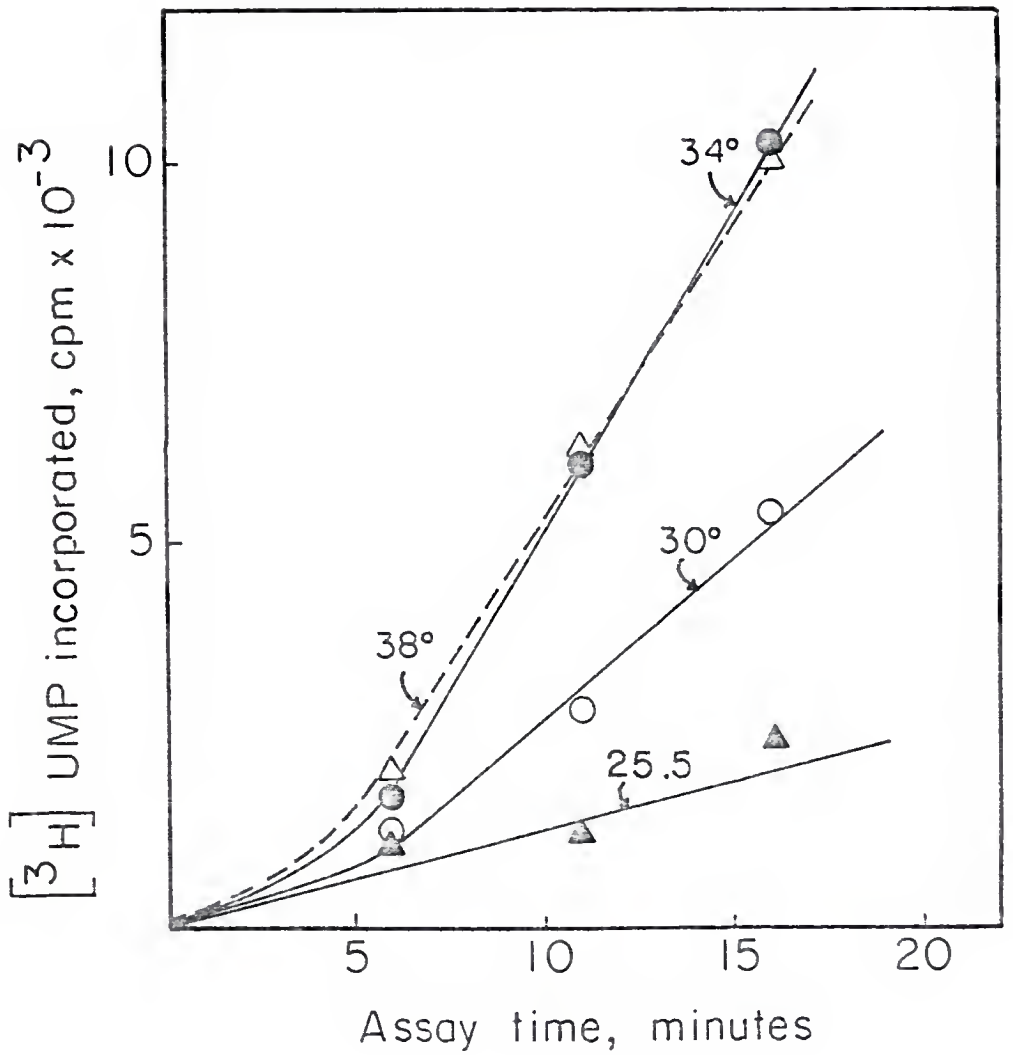
Buffer[a]	pH	% optimal activity[b]
HEPES	7.0	100
	7.5	90
	8.0	87
Sodium acetate	4.0	<2
	4.5	<2
	5.0	<2
PIPES	6.0	2
	7.0	7
	7.5	21
Tris-hydrochloride	7.5	3
	8.0	<2
TES	7.0	30
	7.5	45
	8.0	52
Sodium phosphate	7.0	14
	8.0	<2
Sodium borate	8.5	92
	9.0	10
	9.5	7

[a]The final concentration of each buffer was 40 mM.

[b]Reactions were for 40 min under standard reaction conditions using poliovirion RNA and oligo(U).

Figure 5.

Effect of temperature on the rate of incorporation of labeled substrate into product RNA. Rates of incorporation by the fraction V polymerase were measured at 25.5°, 30°, 34°, and 38°C using poliovirion RNA·oligo(U). Other reaction conditions were as described in Chapter II.


[3H] UMP incorporated, cpm x 10-3
10
5
34°
30°
38°
25.5
5
10
15
20
Assay time, minutes

not further affect the incorporation rates (slope = 830 cpm/min) (Fig. 3). Although the initial incorporation rates were highest at 34°C and 38°C, most reactions were typically carried out at 30°C since the rate remained linear for a longer period of time at this temperature.

Under the reaction conditions used in this study, an oligo(U) primer was required for polymerase activity when poliovirion RNA was used as a template. There was a direct relationship between the amount of product synthesized and the amount of oligo(U) added to the reaction (Fig. 4C). The activity of the purified polymerase reached a maximum value when the ratio of oligo(U) molecules to virion RNA molecules was about 8:1 (Fig. 4C). The amount of oligo(U) necessary for optimal polymerase activity varied depending upon the lot of oligo(U) used and the temperature of the reaction (data not shown). In addition, it was necessary to treat commercial preparations of oligo(U) with alkaline phosphatase to achieve maximal activity (data not shown). Presumably a 3'phosphate remained on many of the oligo(U) molecules and inhibited the polymerase activity. Some partially purified preparations of polymerase (fractions II and III) were active on poliovirion RNA in the absence of an oligo(U) primer (unpublished observations). The amount of product synthesized ranged from 1 to 10% of the amount synthesized when the optimum amount of primer was added to the reaction (data not shown). This activity was lost upon further purification of the enzyme and was not detectable in the purified fraction V polymerase (Fig. 3).

All four ribonucleoside triphosphates were required by the enzyme to copy poliovirion RNA (Table 4). The incorporation of

TABLE 4. Requirement for added ribonucleoside triphosphates.

Reaction condition	Incorporation of [^{3}H]NMP (cpm x 10^{-3})	% of control
[^{3}H]GTP, complete	32.8	100
Minus ATP	4.5	14
Minus CTP	<0.1	<0.3
Minus UTP	<0.1	<0.3
[^{3}H]UTP, complete	28.0	100
Minus ATP	4.3	15
Minus GTP	9.0	32
Minus CTP	3.9	13

NOTE: The reaction mixtures contained fraction III polymerase, poliovirus RNA (2 μg), oligo(U) (0.085 μg), and either [^{3}H]GTP or [^{3}H]UTP as the labeled substrate. The standard reaction conditions were used, except that the unlabeled ribonucleoside triphosphate concentration was 78 μM. The reaction mixtures were incubated at 30°C for 60 min.

[^{3}H]GTP into acid-insoluble product was completely inhibited if either CTP or UTP was not added. Some labeled substrate, however, was incorporated when ATP was deleted from the reaction (Table 4). The sequence at the 3'end of poliovirus RNA, i.e., -CGGAGGpoly(A) (Porter and Fellner, 1978; Kitamura and Wimmer, 1980), suggests that the incorporation of the first [^{3}H]GMP residue into the 5'end of the minus-strand product would be blocked when either CTP or UTP (but not ATP) was deleted from the reaction. The incorporation of [^{3}H]UMP was also inhibited when either ATP, CTP or GTP was deleted from the reaction mixture (Table 4). The residual activity in these reactions probably represents poly(U) synthesis directed by the poly(A) sequence at the 3'end of the template. Under standard reaction conditions, when the Mg^{2+} concentration was 3 mM and when [^{3}H]UTP (45 μM) was used as the labeled substrate, the apparent K_m for the combined nucleotides CTP, ATP and GTP was 25 μM (data not shown).

Discussion

A soluble poly(U) polymerase present in poliovirus-infected cells was purified and was shown to copy poliovirion RNA *in vitro*. The activity on poliovirion RNA was dependent on the presence of oligo(U), the four ribonucleoside triphosphates, and Mg^{2+}. At early stages of the purification (fractions II and III) 1-10% of the control activity was detected when oligo(U) was deleted from the reaction. Lund and Scraba (1979) found similar results in their studies with a soluble RNA polymerase that they isolated from mengovirus-infected cells. They found that a partially purified

preparation of polypeptide E (a 58,000-dalton protein that appears to be the equivalent of p63) exhibited an RNA polymerase activity that was dependent upon mengovirion RNA and oligo(U).

Dasgupta et al. (1979) also reported that partially purified preparations of the poliovirus poly(U) polymerase would copy poliovirion RNA in vitro. In their studies, however, Dasgupta et al. found that the addition of oligo(U) to the polymerase reaction had little stimulatory effect. This may have resulted from the presence of a 3'-terminal phosphate on the oligo(U) used in their studies. We have found that most preparations of oligo(U) require phosphatase treatment to be fully active in the polymerase reaction. Lowe and Brown also reported that a partially purified soluble RNA polymerase which was isolated from FMDV-infected cells could copy FMDV RNA in vitro in the absence of primer. In these studies they did not determine if oligo(U) stimulated polymerase activity (Lowe, P. A., and F. Brown, Virology, in press). Neither the specificity nor the mechanism of initiation of RNA synthesis in the in vitro "unprimed" polymerase reactions has been determined. The initiating activity present in our polymerase preparations at early stages in purification, however, was lost when the enzyme was further purified. This implies that other factors required for the initiation activity were removed or inactivated during polymerase isolation.

The purified polymerase described in this study was very stable. This seems to be contradictory to the early reports that the poliovirus RNA polymerase was unstable. These reports were based on experiments which demonstrated that poliovirus RNA synthesis in

infected cells was dependent upon concurrent protein synthesis (reviewed by Baltimore, 1969). It is possible that the polymerase is required in stoichiometric amounts *in vivo*. Alternatively, other unstable proteins may be required for the initiation of RNA synthesis.

CHAPTER IV
POLYPEPTIDE COMPOSITION OF THE POLYMERASE

Introduction

Only one virus-coded protein was found associated with purified preparations of the poliovirus endogenous RNA replication complex (Lundquist et al., 1974; Flanegan and Baltimore, 1979). This protein was identified by Lundquist et al. as NCVP4 (Mr = 5.8×10^4) and Flanegan and Baltimore as p63 (Mr = 6.3×10^4). Because the two proteins have about the same electrophoretic mobility relative to other poliovirus proteins and have the same enzymatic activity, the variation in molecular weight apparently results from the use of different gel systems. This protein is referred to as p63 in this study. The association of p63 with the elongation activity of the endogenous replication complex strongly suggests that this protein is a component of the poliovirus RNA polymerase.

Etchison and Ehrenfeld (1980) have recently reported that a second viral protein, NCVP4a (Mr $\sim$ 65,000) may also be associated with certain preparations of the endogenous replication complex. Their results suggest that NCVP4a is an unstable precursor of p63. Although further investigation is required to determine if any polymerase activity is associated with NCVP4a, these researchers

suggest the possibility that this protein may be involved in the initiation of RNA synthesis.

In addition to the viral proteins described, several host proteins have also been found in preparations of the replication complex (Butterworth et al., 1976; Lundquist et al., 1974). Dmitrieva et al. (1979) suggested that some of the host proteins associated with the EMCV replication complex may be required for the replication of viral RNA. The role of host proteins in poliovirus RNA synthesis, however, has yet to be determined.

To further examine the structure of the poliovirus RNA polymerase, it was necessary to isolate the polymerase as a soluble enzyme that was dependent on an exogenous RNA template for activity. When the soluble poly(A)-dependent poly(U) polymerase was first isolated by Flanegan and Baltimore (1979), two viral proteins, p63 and NCVP2, were found to copurify with the activity. Because this enzyme sedimented at 7 S in a glycerol gradient, it was suggested that the enzyme might be a complex of p63 and NCVP2 or a dimer of one of the two proteins (Flanegan and Baltimore, 1979). Additional evidence has suggested that both of these proteins might be required components of the poliovirus RNA-dependent RNA polymerase (Bowles and Tershak, 1978; Korant, 1975; Lundquist et al., 1974; Lundquist and Maizel, 1978).

To determine if both p63 and NCVP2 were required for polymerase activity, we analyzed the viral proteins associated with the purified soluble polymerase. The in vitro polymerase assay used in this study employed an oligo(U) primer and a poliovirion RNA template. We found that p63 was the only viral protein that copurified with

the polymerase activity during gel filtration on Sephacryl S-200. No polymerizing activity was found associated with NCVP2 when it was separated from p63. All host proteins still present were recovered in submolar amounts relative to p63. The purified enzyme sedimented at about 4 S on a glycerol gradient, and thus appeared to be a monomer of p63 and identical to the polymerase associated with the endogenous RNA replication complex.

Results

Viral Proteins Present during Polymerase Purification

To determine which virus-specific polypeptides were required for polymerizing activity on an exogenously added poliovirion RNA template, we purified the soluble poliovirus RNA polymerase from infected cells grown in the presence of [^{35}S]methionine. The virus-specific proteins present at each stage of purification were examined by SDS-polyacrylamide gel electrophoresis and autoradiography. Because poliovirus infection inhibits the synthesis of host proteins, only virus-specific proteins were labeled and detected in these experiments (Ehrenfeld and Lund, 1977). Thus, purification of the polymerase allowed us to specifically determine which virus-specific proteins copurified with polymerase activity. The enzyme was purified as a poly(U) polymerase using a poly(A) template complexed to an oligo(U) primer. At various stages of purification the enzyme was tested for its ability to copy a poliovirion RNA template complexed to an oligo(U) primer.

Initial fractionation of the poliovirus RNA polymerase from other soluble proteins in the cytoplasm of infected cells was

achieved by ammonium sulfate precipitation and by phosphocellulose chromatography as described in Chapter III. Polyacrylamide gel electrophoresis showed that four major viral proteins were still present at this stage of purification (Fig. 6, lane 4). During this initial purification of the polymerase, the percentage of the total radioactivity recovered in NCVP2 and p63 increased significantly, whereas the amount of radioactivity present in the two smaller proteins (p45 and NCVPY) remained about the same (Fig. 6, lanes 1-4). Additional purification of the enzyme was necessary to determine which proteins were required for polymerase activity.

Affinity chromatography on poly(U) Sepharose was previously shown by Dasgupta et al. (1979) to be a useful procedure for purifying the poliovirus RNA polymerase. Adsorption of the fraction III enzyme to poly(U) Sepharose and batch elution with 0.25 M KCl resulted in an additional twofold purification of the polymerase (data not shown). When each fraction containing polymerase activity was analyzed for viral proteins by SDS-polyacrylamide gel electrophoresis, the same four proteins (NCVP2, p63, p45, NCVPY) were found to have coeluted with the activity (data not shown). The relative amounts of each viral protein were essentially the same as that found after phosphocellulose chromatography (Fig. 6, lane 4). Therefore, chromatography on poly(U) Sepharose did not prove useful in fractionating these four proteins relative to each other and to the peak of polymerase activity.

Further purification of the polymerase by a combination of gel filtration through Sephacryl S-200 and by hydroxylapatite chromatography resulted in a fractionation of these four viral

proteins relative to the activity of the polymerase (Fig. 6, lanes 5 and 6). The major viral protein component of the purified enzyme was p63 (Fig. 6, lane 6). In addition, it was clear that the fraction of the total radioactivity recovered in p63 relative to the other viral proteins increased at each purification step (Fig. 6, lanes 1-6). These results suggested that p63 was the only viral protein component of the polymerase.

Gel Filtration on Sephacryl S-200

Although p63 was the major viral protein component of the purified polymerase, a small amount of NCVP2 and a trace of NCVPY were still present. Thus, it was not possible to definitevely state that p63 was the only viral protein required for polymerase activity. Because gel filtration enriched only p63 and diminished the quantity of NCVP2 and the other viral proteins, we analyzed the relative elution positions of the polymerase and the labeled viral proteins after chromatography on Sephacryl S-200. The column fractions were assayed for poly(U) polymerase activity and for polymerase activity on poliovirion RNA. The two activities were found to copurify in the exact same position (data not shown). To determine which of the viral proteins eluted with these activities, an equal portion of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis for the presence of ^{35}S-labeled proteins (Fig. 7). P63 was the only viral protein that precisely eluted with the peak of polymerase activity (Fig. 7). Noncapsid viral protein 2 eluted ahead of p63, as did p45 and NCVPY, in a region where no polymerizing activity was detected (Fig. 7). In addition, the activity of the polymerase was not enhanced by mixing equal amounts of fraction number 36 (the

Figure 6.

Polyacrylamide gel electrophoresis of poliovirus RNA polymerase at each stage of purification. The polymerase was purified from infected cells grown in the presence of [^{35}S]methionine (Table 1). The labeled proteins present at various stages of purity were analyzed by SDS-polyacrylamide gel electrophoresis as follows: lane 1, cytoplasmic extract; lane 2, 200,000 x g supernatant (fraction I); lane 3, $(NH_4)_2SO_4$ precipitate (fraction II); lane 4, phosphocellulose (fraction III); lane 5, Sephacryl (fraction IV); lane 6, hydroxylapatite (fraction V). A constant amount of labeled protein (900 cpm of acid insoluble material) was loaded in lanes 1 through 6. Lane 7 contained ^{125}I-labeled phosphorylase b (phos b), bovine serum albumin (BSA), catalase (cat), and ovalbumin (OA).

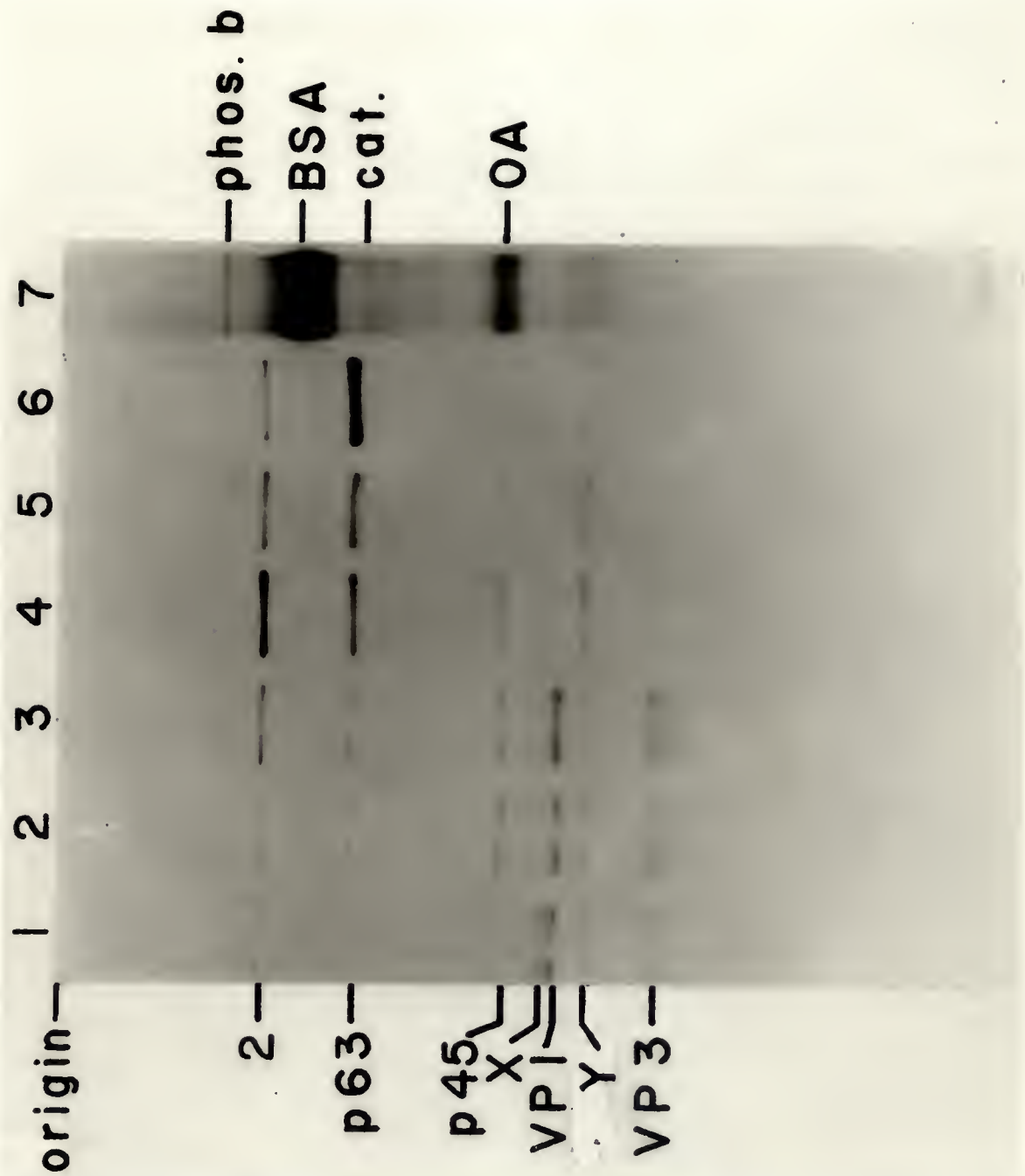
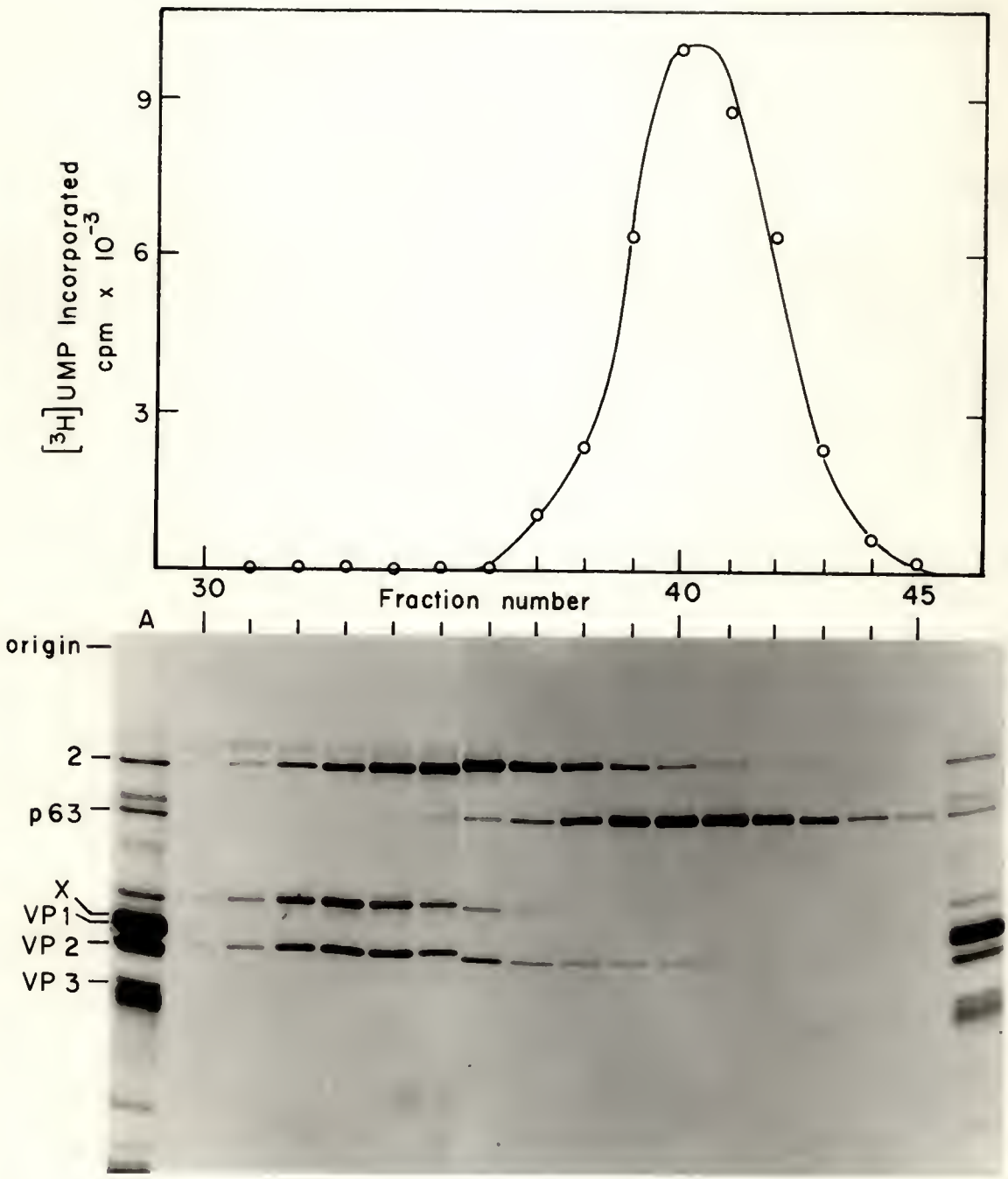

origin
1
2
3
4
5
6
7
phos. b
BSA
cat.
OA
2
p63
p45
X
VP1
Y
VP3

Figure 7.

Gel filtration on Sephacryl S-200 (top) and polyacrylamide gel electrophoresis (bottom) of poliovirus RNA polymerase. The polymerase was isolated from cells grown in the presence of [^{35}S]methionine by high-speed centrifugation, $(NH_4)_2SO_4$ precipitation, and phosphocellulose chromatography (Table 1). After phosphocellulose chromatography, the peak fractions of polymerase activity were pooled, concentrated fourfold with solid sucrose, and chromatographed on a Sephacryl S-200 column (1.6 x 63 cm). A portion of each fraction was assayed for polymerase activity on poly(A)·oligo(U) (data not shown) and on poliovirion RNA·oligo(U) (top). The labeled proteins in fractions 30 to 35 were analyzed on the gel to the left and fractions 36-45 were analyzed on the gel to the right. Lane A contained ^{35}S-labeled poliovirus cytoplasmic proteins. About 40% of the input activity was recovered in the combined peak fractions.


[3H]UMP Incorporated
cpm x 10-3
9
6
3
30
Fraction number
40
45
A
origin
2
p63
X
VP1
VP 2
VP 3

peak of NCVP2) with fraction number 40 (the peak of p63 and polymerase activity) (data not shown). Consistent with this result is the fact that the peak of activity was symmetrical (Fig. 7). These results argue that the viral polypeptide associated with the purified polymerase was p63 and not NCVP2, and that NCVP2 alone had no polymerase activity and was not required for activity under the assay conditions tested.

Size of the Polymerase

We attempted to estimate the size of the polymerase by determining its relative elution volume during gel filtration. A Sephacryl S-200 column (1.6 x 180 cm) was prepared and was calibrated with five protein molecular weight standards which were well resolved by the column (Fig. 1C). Unexpectedly, the polymerase eluted very late from the column in a position that corresponded to a molecular weight of 20,000 (Fig. 1C). Increasing the KCl concentration in the column buffer to 0.5 M had no effect on this profile. Because the enzyme was expected to elute at a position corresponding to a molecular weight of 63,000 or larger, the polymerase may have had a special affinity for the Sephacryl resin which retarded its mobility.

Because the poliovirus polymerase ran anomalously on Sephacryl S-200, it was not possible to determine its size by this method. For this reason the size of the polymerase was analyzed at various stages of purity by glycerol gradient centrifugation. Prior to chromatography on phosphocellulose, the polymerase sedimented broadly at about 6 S (Fig. 8A and Flanegan and Van Dyke, 1979) and this suggested that the polymerase was not a monomer of p63 at this stage of purity. In contrast, the polymerase sedimented as a sharp

peak at 4 S after phosphocellulose chromatography (Fig. 8B) as would be expected for a monomer of p63. We do not yet understand the composition of the 6 S polymerase, nor do we know exactly what causes its conversion to the 4 S form. We do know that the conversion is a result of chromatography on phosphocellulose and is not a function of the salt concentration, protein concentration, the presence of Nonidet P40 or treatment with the reducing agent DTT (data not shown).

Host-Coded Polypeptides and Polymerase Activity

To determine if any host-coded proteins had copurified with the polymerase in quantities similar to p63, we analyzed the fraction IV (Table 1) polymerase on an SDS-polyacrylamide gel and stained the protein bands with Coomassie brilliant blue. The gel was dried and autoradiographed to compare labeled viral proteins with stained bands. The major labeled viral protein present was p63, although small amounts of NCVP2 and NCVPY were also present (Fig. 9, lane 5). Only three stained bands were detected in the same lane of the gel (Fig. 9, lane 4). One band migrated at the exact same position as p63. The other stained bands were ovalbumin and its breakdown product which were present in the column buffer as carrier protein (data not shown). The staining procedure was not sensitive enough to detect the trace amounts of NCVP2 and NCVPY that were present. All of the host proteins that were present at the early stages of purification (Fig. 9, lanes 1-3) could not be detected in the purified enzyme (Fig. 9, lane 4). Thus, we concluded from this result that there were no host proteins present in the same molar amount as p63 in the purified enzyme preparation.

Figure 8.

Glycerol gradient centrifugation of the poliovirus RNA polymerase. The polymerase was analyzed by centrifugation on a 15-30% glycerol gradient before (A-fraction II, Table 1) and after (B-fraction III, Table 1) chromatography on phosphocellulose as described in Chapter II. Bovine serum albumin (BSA) was run in a parallel gradient with both samples as a sedimentation marker.

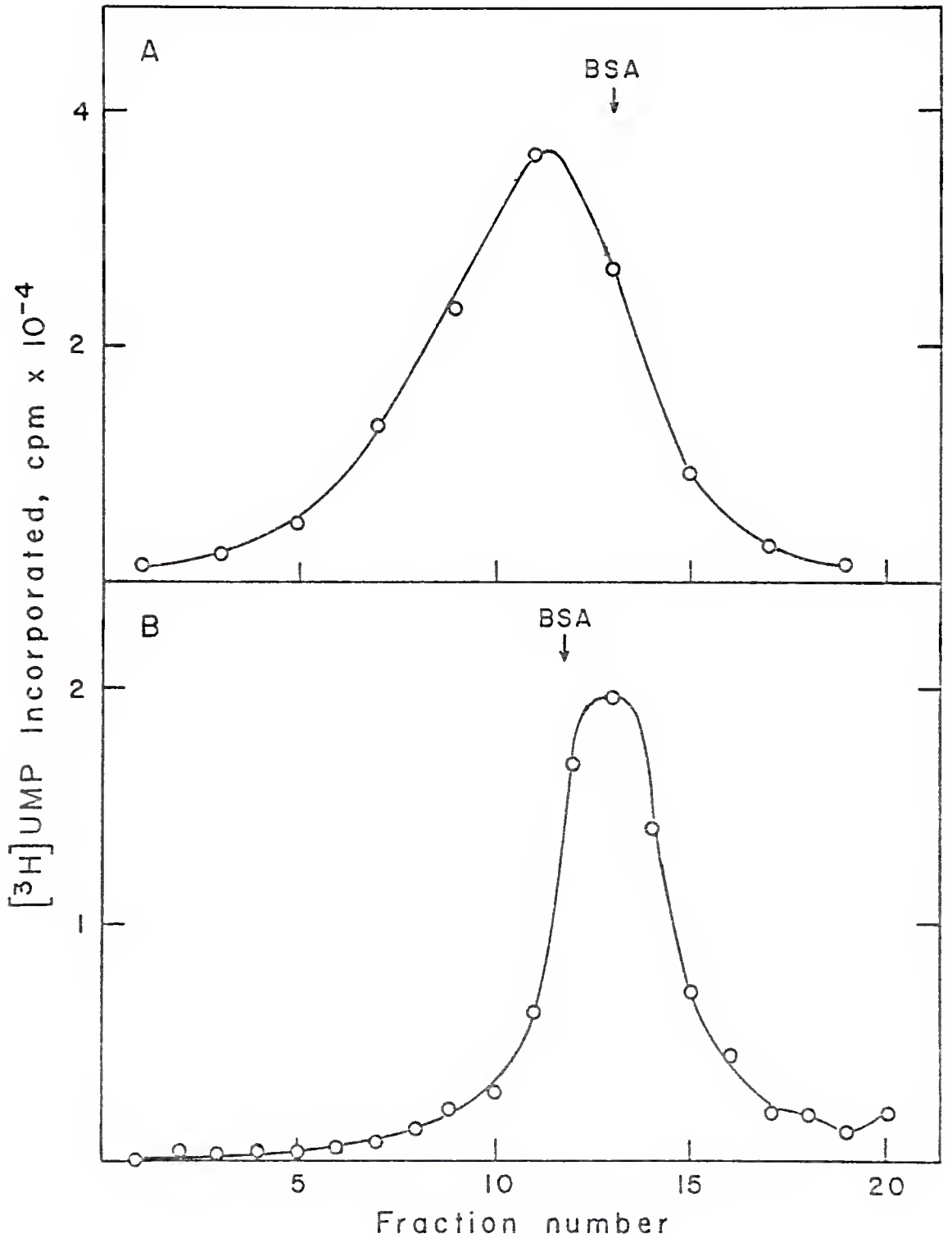
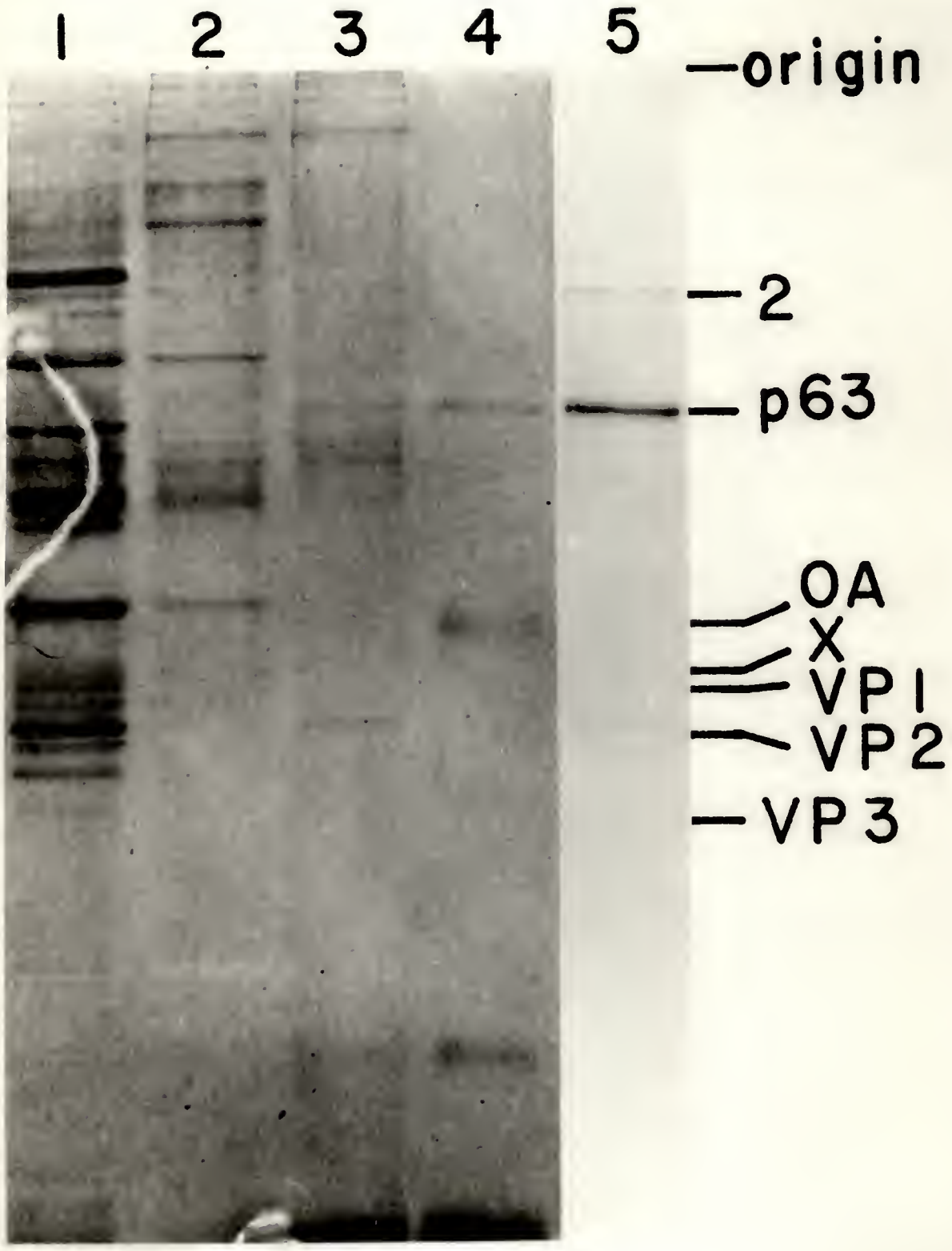

A
BSA
4
2
B
BSA
2
1
[3H]UMP Incorporated, cpm x 10-4
5
10
15
20
Fraction number

Figure 9.

Protein composition of poliovirus RNA polymerase determined by staining and autoradiography after polyacrylamide gel electrophoresis. The polymerase was isolated from infected cells grown in the presence of [^{35}S]methionine (Table 1). The proteins present at various stages of purity were analyzed by SDS-polyacrylamide gel electrophoresis and by staining with Coomassie brilliant blue. Polymerase fractions were analyzed as follows: lane 1, 200,000 x g supernatant (fraction I); lane 2, $(NH_4)_2SO_4$ precipitate (fraction II); lane 3, phosphocellulose (fraction III); lane 4, Sephacryl S-200 (fraction IV). The labeled proteins present in the fraction IV enzyme (lane 4 above) were detected by autoradiography of the same area of the gel (lane 5).


1
2
3
4
5
origin
2
p63
OA
X
VP1
VP2
VP3

Two-Dimensional Gel Electrophoresis

We felt that it was important to analyze the structure of the polymerase by two-dimensional gel electrophoresis in order to characterize the polymerase protein in terms of its isoelectric point as well as its molecular weight. In addition, we wanted to determine if more than one viral protein might be migrating at a molecular weight of 63,000 during polyacrylamide gel electrophoresis. Therefore, the purified polymerase was analyzed by isoelectric focusing in the presence of urea in the first dimension and by SDS-polyacrylamide gel electrophoresis in the second dimension. When the purified polymerase (Table 1, fraction V) was analyzed by this method, only one viral protein was found in the 63,000d region of the gel and this protein had an isoelectric point of 7.5 (Fig. 10A). A trace amount of NCVP2 was also detected with an isoelectric point of 7.7. The position of p63 relative to other viral proteins in the two-dimensional gel was also determined (Fig. 10B). This served to compare the isoelectric point of p63 relative to that of the other viral proteins (Table 5).

Discussion

These results indicate that p63 is the only polypeptide component of a soluble RNA-dependent RNA polymerase that is present in the cytoplasm of poliovirus-infected cells. The purified polymerase did not appear to require the presence of any additional virus-coded or host-coded proteins for activity and was found to sediment

Figure 10.

Two-dimensional gel electrophoresis of poliovirus RNA polymerase. (A) The purified polymerase (fraction V, Table 1) was isolated from infected cells grown in the presence of [^{35}S]methionine and was analyzed by two-dimensional gel electrophoresis. The ^{35}S-labeled poliovirus proteins present in fraction II (Table 1) were electrophoresed as marker proteins in the second dimension only (lane 2). A lighter exposure of lane 2 is shown in lane 1. (B) Two-dimensional analysis of the fraction II proteins. Molecular weights were assigned to the proteins in (B) by using the values determined for NCVP2, p63, and VP3 in Fig. 6. The pH gradient across the gel was determined as described in Chapter II.

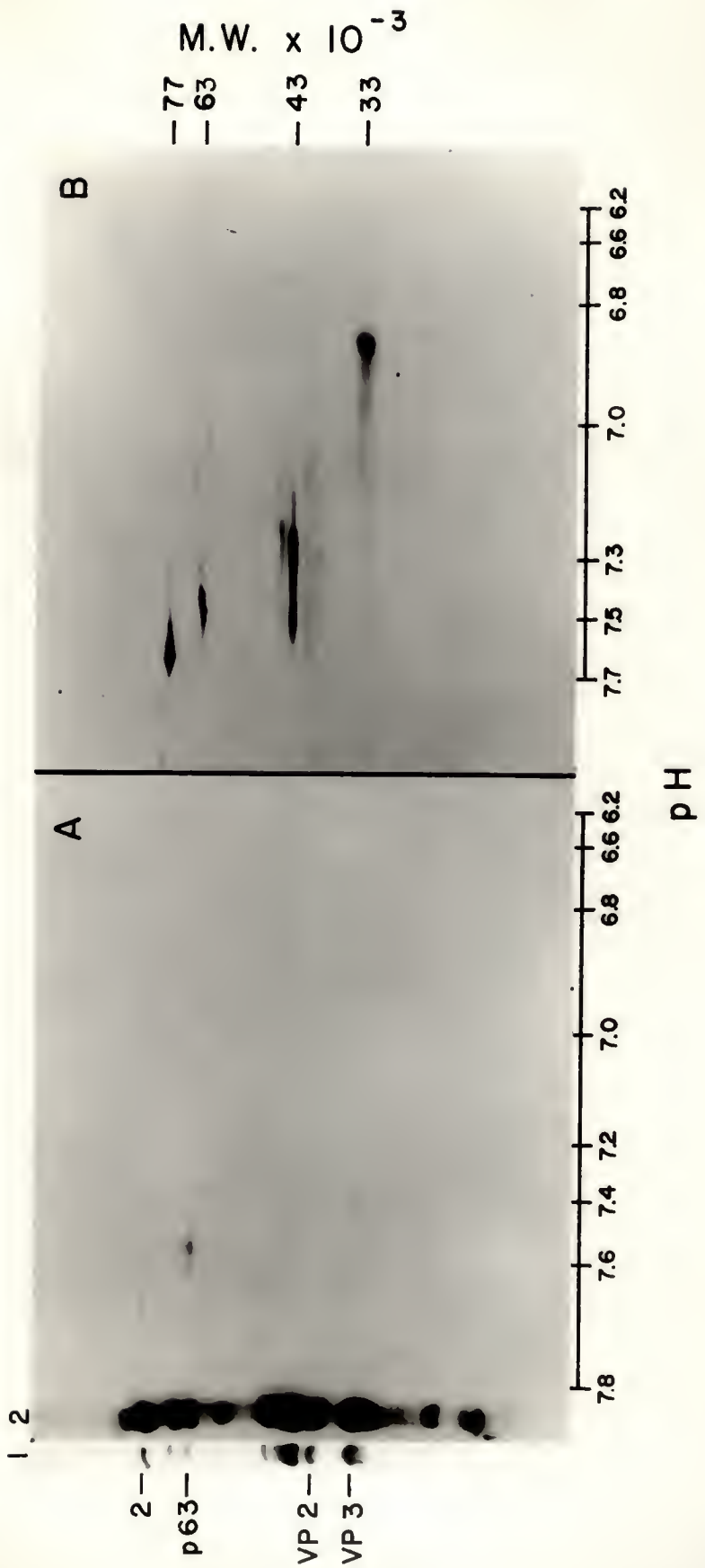

M.W. x 10⁻³
77
63
43
33
B
A
6.2
6.6
6.8
7.0
7.3
7.5
7.7
7.8
7.6
7.4
7.2
pH
1
2
2—
p63—
VP 2—
VP 3—

TABLE 5. Isoelectric point and molecular weight of poliovirus proteins separated by two-dimensional gel electrophoresis.

Molecular Weight[a]	Isoelectric Point[b]
77,000 (NCVP2)	7.65
65,000	7.1
63,000 (p63)	7.45
60,000	7.05
46,000	7.1 - 7.5
43,000	7.15 - 7.55
41,000	7.05 - 7.65
38,000	7.35
33,000 (VP3)	6.95

[a]Assigned based on the position of the proteins in Fig. 10B relative to the identified viral proteins, NCVP2, p63 and VP3. The molecular weight of these reference proteins was calculated based on their mobility compared with molecular weight standards as shown in Fig. 6.

[b]Determined from position in the two-dimensional gel shown in Fig. 10B.

at about 4 S in a glycerol gradient. These findings suggest that p63, in monomer form, is responsible for the purified polymerase activity.

Results from previous studies suggested that both p63 and NCVP2 might be required for polymerase activity (Bowles and Tershak, 1978; Flanegan and Baltimore, 1979; Korant, 1975). Therefore, to determine if one or if both proteins were required for polymerase activity, it was necessary to separate p63 from NCVP2. This was achieved by gel filtration in Sephacryl S-200. The results showed that NCVP2 alone had no associated polymerase activity and that it was not required by p63 for polymerase activity *in vitro*. Similar results have now been obtained for FMDV by Lowe and Brown (Lowe, P. A., and F. Brown, Virology, in press). They have shown that the FMDV RNA polymerase copurifies with the FMDV protein, p56. The precursor of this protein, p72, was not required for polymerase activity and had no associated polymerase activity.

Our findings were consistent with the previous work in which it was demonstrated that p63 was the only virus-specific protein associated with the endogenous RNA replication complex (Flanegan and Baltimore, 1979). P63 is apparently the same protein that was designated as NCVP4 by Lundquist *et al.* (1974) and Butterworth *et al.* (1976) in their studies on the endogenous RNA replication complex. In other reports, however, viral proteins that may not be the polymerase protein have been designated as NCVP4 (Bowles and Tershak, 1978; Villa-Komaroff *et al.*, 1974). To help clarify this situation, we have analyzed the polymerase protein by two-dimensional gel electrophoresis. This provides two physical parameters for the identification of the polymerase protein. The protein's isoelectric

point was about 7.5 in the presence of urea and its apparent molecular weight was 63,000. In addition, the position of this protein in the two-dimensional gel relative to NCVP2 and other viral proteins should prove useful in identifying this protein in future studies.

We have made no conclusions about what proteins may be required to initiate poliovirus RNA replication in vivo. In this study, we have only assayed for activity on a poliovirion RNA template when oligo(U) was used as a preformed primer. Initiation of RNA synthesis in vivo may require the presence of additional virus-coded and host-coded proteins. Before chromatography on phosphocellulose, the polymerase sediments at about 6 S. This suggests that p63 is complexed with another factor (or factors) prior to chromatography on phosphocellulose. This factor has not been identified and it is apparently not required for activity in our in vitro assay, but it may be required to initiate RNA synthesis in the absence of an added primer. One protein factor that has been related to the initiation of viral RNA synthesis is a host-coded protein that was described by Dasgupta et al. (1980). This protein stimulated poliovirus RNA polymerase activity in vitro. Recent evidence suggests that this host-coded protein may play a role in initiating RNA synthesis because the protein stimulates polymerase activity in the absence of oligo(U) but not in its presence (Dasgupta et al., 1980). However, additional studies are required to define the mechanism by which this protein stimulates polymerase activity. It has also been proposed that the protein which is covalently linked to the 5'-terminal end of poliovirion RNA (i.e., VPg) may play a role in initiating viral RNA synthesis (Flanegan et al., 1977; Nomoto

et al., 1977). Because NCVP1b is apparently the precursor to p63, a protease, and VPg, it has also been suggested that NCVP1b may serve to initiate RNA synthesis in vivo (Palmenberg et al., 1979 and Kitamura et al., 1980). The proposal in this model is that the in situ cleavage of NCVP1b by the protease yeilds the active polymerase (p63) and the primer (VPg). These models are still speculative and the mechanism by which poliovirus RNA synthesis is initiated is not known.

CHAPTER V
CHARACTERIZATION OF THE PRODUCT SYNTHESIZED *IN VITRO* BY THE PURIFIED POLYMERASE

Introduction

The soluble polymerase that was isolated from the cytoplasmic extracts of infected cells was shown to use both poly(A) and poliovirion RNA as templates. In both cases an oligo(U) primer was required for activity by the purified polymerase. When virion RNA was used as a template, it was assumed that the oligo(U) primer was hybridizing with the 3'-terminal poly(A) sequence and that product RNA synthesis was, therefore, initiating at the 3' end of the template. If this is the case, then it should be possible to synthesize a full-sized RNA product in the *in vitro* reaction. This of course assumes that the purified polymerase has an elongating activity that is similar to the enzyme that functions *in vivo* and that it can make complete copies of the template RNA.

In this study, we have characterized the product RNA which was synthesized by the purified polymerase in the *in vitro* reaction. When virion RNA was used as a template, the product RNA was shown to be heteropolymeric and complementary to the template RNA. Under appropriate reaction conditions, the product was also shown, by electrophoresis in CH_3HgOH-agarose gels, to be full-sized and covalently linked to the oligo(U) primer. Changes in the reaction conditions were found to have a significant effect on the elongation

rates, initiation rates, and the fraction of the total product RNA that was full-sized. Under optimal conditions, we found that most of the product RNA synthesized in the *in vitro* reaction was full-sized, and that about 6 min were required to synthesize a complete copy of the virion RNA template.

Results

Product Length

The size of the product RNA synthesized by the purified polymerase was examined by electrophoresis in agarose gels containing 5 mM methylmercury hydroxide (CH_3HgOH) as a denaturing agent. When electrophoresed in a 1% agarose gel lacking CH_3HgOH, double-stranded (ds) poliovirus RNA (20 S) had a much slower mobility than that of single-stranded (ss) virion RNA (35 S) (Fig. 11A, compare lanes 1a and 5a). The mobility of the product RNA on agarose gels lacking CH_3HgOH was similar to that of dsRNA, suggesting that the product RNA was still hybridized to the virion RNA template (Fig. 11B, lanes 4b and 3b). Denaturation of the product from the template was therefore necessary for accurate size determination.

When dsRNA was electrophoresed in a 1% agarose gel containing CH_3HgOH, it migrated with the same mobility as poliovirus ssRNA (Fig. 12B, compare lanes 1 and 2). Analysis of labeled product RNA in a CH_3HgOH-agarose gel showed that some genome-sized product RNA was synthesized in the *in vitro* reaction (Fig. 12A, lanes 1-4). When synthesized at 3 mM Mg^{2+} (the optimum Mg^{2+} concentration for incorporation), a large amount of the product RNA was subgenome-length and many discrete size classes were present

Figure 11.

Nondenaturing agarose gel electrophoresis of poliovirus RNAs and product RNA. Poliovirus ssRNA (35 S), dsRNA (20 S), and product RNA were electrophoresed in a 1% agarose gel which did not contain a denaturing agent. (A) the gel was stained with ethidium bromide to detect unlabeled RNA, and (B) autoradiographed to detect the labeled 20 S RNA and product RNA. The samples were analyzed as follows: lane 1, ^{14}C-labeled poliovirus dsRNA (20 S); lane 2, unlabeled 18 S and 28 S HeLa ribosomal RNAs; lane 3, ^{32}P-labeled product RNA which was synthesized at 7 mM Mg^{2+}; lane 4, ^{32}P-labeled product RNA which was synthesized at 3 mM Mg^{2+}; lane 5, unlabeled virion RNA (35 S). The stained bands in lanes 3a and 4a represent the excess 35 S virion RNA template which was present in the reaction mixture.

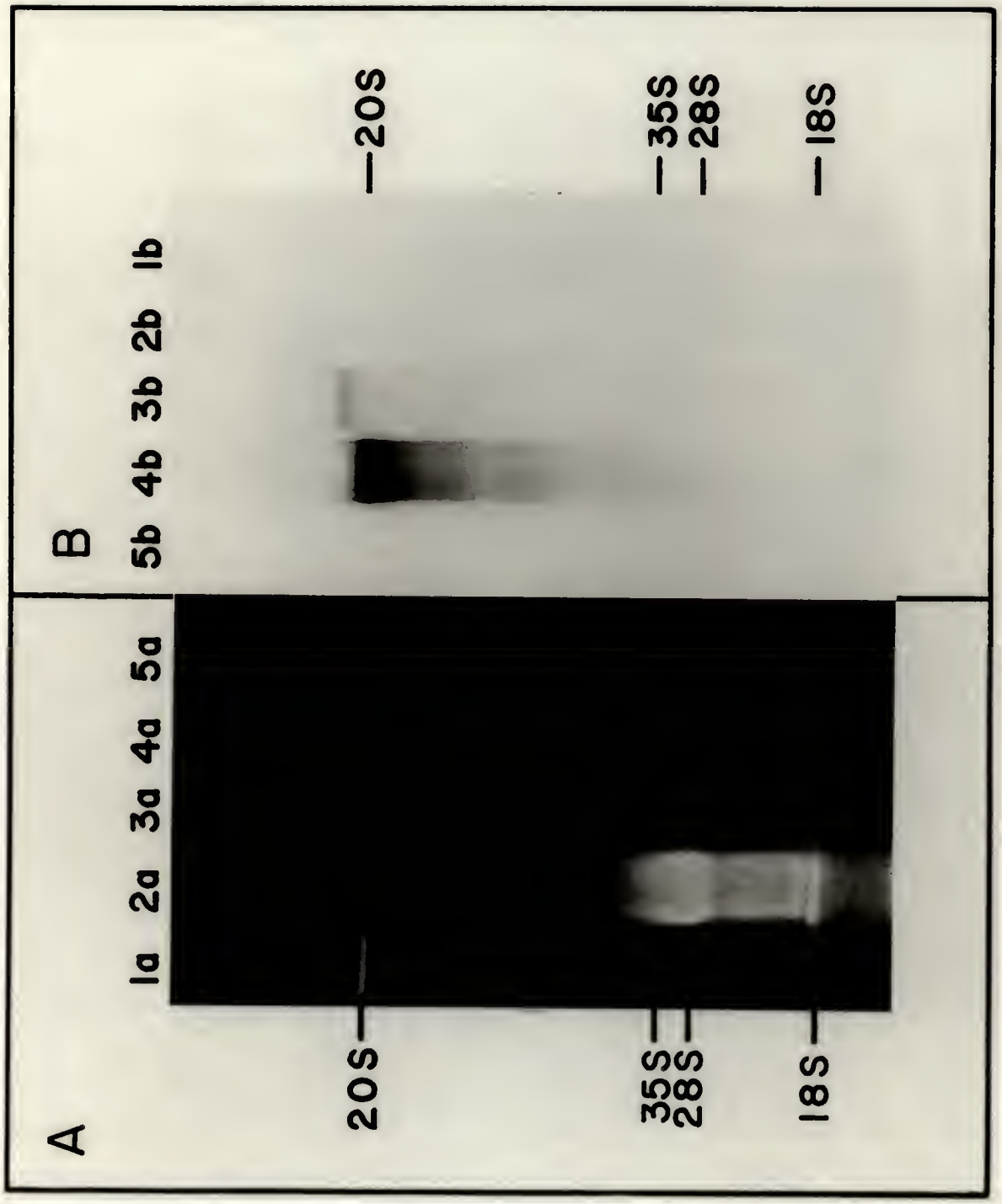
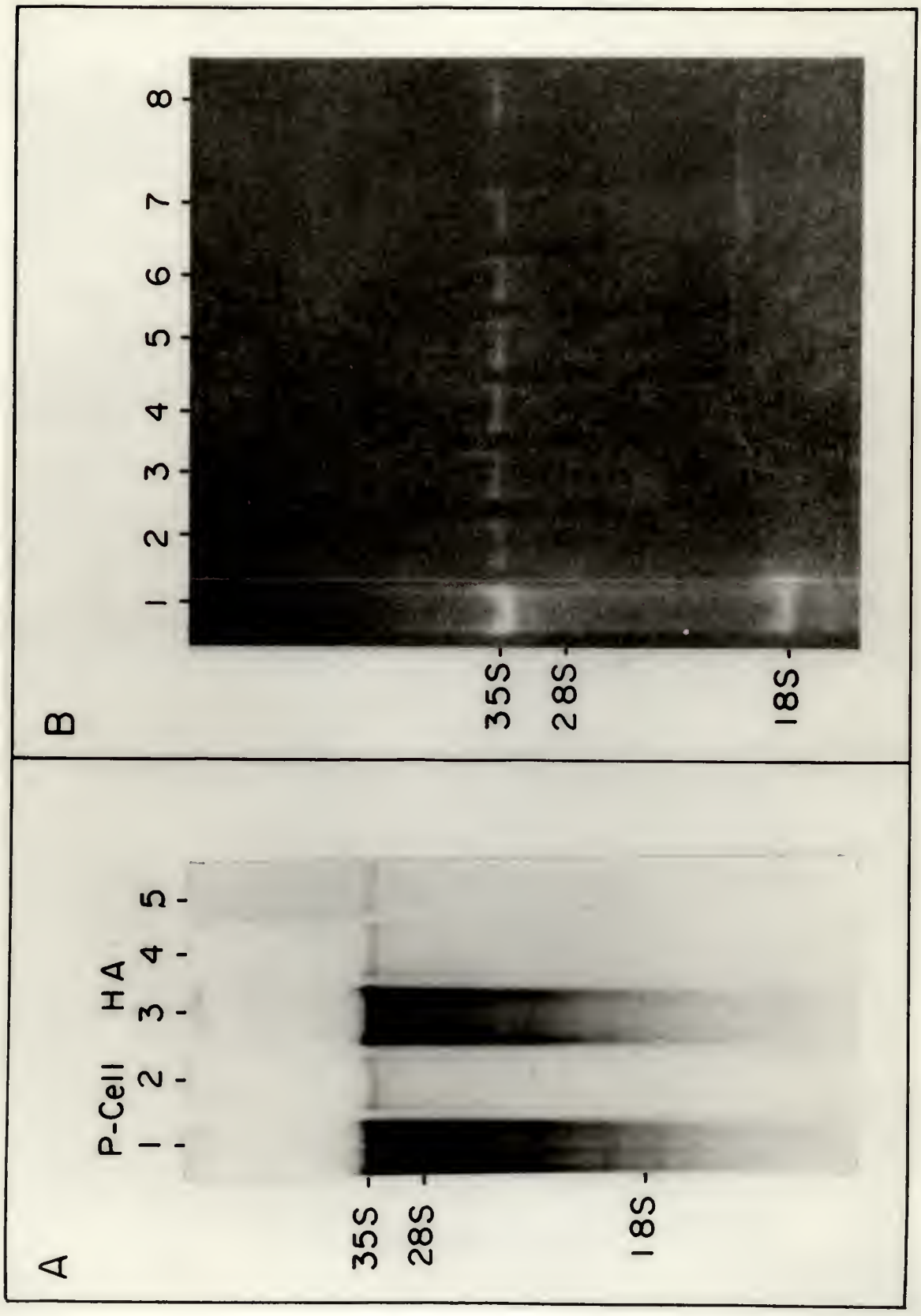

A
1a 2a 3a 4a 5a
20S
35S
28S
18S
B
5b 4b 3b 2b 1b
—20S
—35S
—28S
—18S

Figure 12.

Methylmercury hydroxide agarose gel electrophoresis of product RNA. Labeled product RNA was isolated from the reactions and electrophoresed in a 1% agarose gel containing 5 mM CH_3HgOH as described in Chapter II. (A) The gel was autoradiographed and the samples were analyzed as follows: Lanes 1 and 2, labeled product RNA synthesized by the fraction III polymerase at 3 mM and 7 mM $Mg(CH_3COO)_2$, respectively; lanes 3 and 4, labeled product RNA synthesized by the fraction V polymerase at 3 mM and 7 mM $Mg(CH_3COO)_2$, respectively, lane 5, poliovirus dsRNA labeled *in vivo* with [^{14}C]uridine. (B) A similar gel containing product RNA was stained with ethidium bromide. The samples were analyzed as follows: lane 1, poliovirus dsRNA and 18 S HeLa ribosomal RNA markers; lane 2, poliovirus 35 S RNA marker; lane 3, poliovirus 35 S RNA which had been incubated with the reaction mixture lacking the polymerase; lanes 4 through 8, poliovirus 35 S RNA which had served as template for the synthesis of product RNA in standard reactions containing fraction V polymerase.


A
P-Cell HA
1 2 3 4 5
35S
28S
18S
B
1 2 3 4 5 6 7 8
35S
28S
18S

(Fig. 12A, lanes 1 and 3). If the Mg^{2+} concentration was increased from 3 mM to 7 mM, the majority of the product synthesized was full-length (Fig. 12A, lanes 2 and 4). The effect of the Mg^{2+} concentration, as well as other reaction parameters, on product synthesis will be described in more detail later.

The quality of the product was the same whether synthesized by the less pure fraction III enzyme (Fig. 12A, lanes 1 and 2) or the purified fraction V enzyme (Fig. 12A, lanes 3 and 4). Both polymerase preparations were free of detectable ribonuclease activity. After one hour of incubation with the polymerase, the 35 S virion RNA template was still intact (Fig. 12B, lanes 4-8).

Nearest Neighbor Analysis

The composition of the product was determined by conducting a nearest neighbor analysis. The product RNA was synthesized in the presence of either [α^{32}P]GTP or [α^{32}P]UTP in reactions which yielded mostly full-length molecules (i.e., at 7 mM Mg^{2+}). The labeled product RNA was digested with either KOH or RNases T1, T2 and A which resulted in a transfer of the [α^{32}P)PO_4 from the nucleotide substrate to its 5'-"nearest neighbor" in the product. Four labeled ribonucleoside monophosphates were recovered from the digested product RNA (Table 6), and this clearly indicates the heteropolymeric nature of the RNA synthesized in the *in vitro* reaction.

The nearest neighbor frequencies for GMP in the product RNA agree well with the frequencies predicted for poliovirus minus-strand RNA (Table 6). These predicted frequencies were calculated based on the nucleotide sequence and base composition of plus-strand poliovirion RNA (V. Racaniello and D. Baltimore, personal

communication). The most outstanding feature of these frequencies is the low percentage of CpG present in the poliovirus RNA. It is interesting that the frequency of CpG is also much lower than the other nucleotide pairs in eukaryotic, but not prokaryotic, DNAs (Subak-Sharpe et al., 1966).

Complementarity of Product RNA

The complementarity of the product RNA was examined by testing its sensitivity to RNase digestion before and after hybridization with excess virion RNA. Untreated product RNA was about 90% resistant to RNase treatment (Table 7). This suggested that most of the product RNA was still in a duplex structure with the template RNA after a one hour reaction. When the RNA was heated at 100°C for 10 min and quickly chilled, about 35% of the product RNA was RNase resistant (Table 7). If the RNA was allowed to self-anneal at 50°C for one hour, about 60% of the product was resistant to RNase treatment (Table 7). This value could be driven to 100% under more stringent hybridization conditions (data not shown). Therefore, the above value probably represents the basal level, under the conditions used, of product RNA which annealed to the template RNA used in the in vitro reaction. When the product RNA was annealed with excess unlabeled poliovirion RNA, it was 99% resistant to RNase digestion. Annealing the product with HeLa cytoplasmic RNA had no effect (Table 7). Thus, these results indicate that the product RNA synthesized in vitro by the purified polymerase was complementary to the poliovirion RNA template.

TABLE 6. Nearest neighbor analysis of product RNA synthesized *in vitro* by the poliovirus RNA polymerase.

3'nucleotide	% of Total[a] ([^{32}P]UMP)		% of Total[a] ([^{32}P]GMP)		% of Total (GMP)[b]
	KOH	RNase	KOH	RNase	predicted
UMP	43.3	39.2	39.5	39.1	31.3
GMP	22.7	20.4	23.2	24.6	26.0
AMP	17.4	19.0	27.3	25.9	30.0
CMP	16.6	21.4	10.0	10.4	12.6

NOTE: The product RNA was labeled in the *in vitro* reaction with either [α^{32}P]UTP or [α^{32}P]GTP as indicated above. The labeled product RNA was then digested with either KOH or RNases T1, T2, and A as specified in the table.

[a]The values are expressed as the percent of the total cpm recovered in all four 3'mononucleotides.

[b]The values were calculated based on the sequence and base composition of poliovirion RNA (V. Racaniello and D. Baltimore, personal communication).

TABLE 7. Resistance of product RNA to RNase digestion before and after annealing with excess poliovirion RNA.

Treatment[a]	RNase Resistance (%)[b]
None	90
Boiled and quickly chilled	35
Boiled and annealed with no RNA	64
Boiled and annealed with HeLa RNA	64
Boiled and annealed with poliovirion RNA	99

[a]The preparation and treatment of product RNA were described in Chapter II.

[b]The results are expressed as the percentage of RNase-resistant RNA which was present in each sample.

Elongation and Initiation Rates for RNA Synthesis

The elongation and initiation rates of RNA synthesis in the in vitro polymerase reaction were determined. These rates were calculated by measuring the size of the product RNA as a function of time and by measuring the total amount of labeled substrate incorporated into the product RNA at specified reaction times. The size of the largest product RNA was determined by electrophoresis of the labeled product on CH_3HgOH-agarose gels after specified reaction times (Fig. 13A). It was clear that the subgenome-sized product RNA that was present at early reaction times was a result of synthesis and not degradation, because the input template RNA remained intact during the entire reaction (Fig. 13B).

The polymerase was assayed under various conditions of pH, Mg^{2+} concentration, and temperature. Initial observations indicated that the rate of elongation was greatest at pH 8.0 and 7 mM Mg^{2+} (Fig. 15). Under these conditions, increasing the temperature from 30°C to 37°C increased the elongation rate less than twofold (data not shown). Because optimal incorporation had previously been obtained at pH 7.0 and 3 mM Mg^{2+} (Chapter III), incorporation and elongation rates from the same reactions were compared under various conditions of pH and Mg^{2+} concentration. The results indicate that when conditions were maximal for elongation rates (pH 8.0, 7 mM Mg^{2+}; Fig. 15A), incorporation was minimal (Fig. 15B). The reverse was also true. Thus, the optimum elongation and initiation rates for RNA synthesis were achieved under different reaction conditions. The maximum elongation rate measured in this study (at 7 mM Mg^{2+}, pH 8.0; Fig. 15A) was approximately 700

nucleotides per minute, which resulted in the synthesis of full-length product RNA molecules in approximately ten minutes. In other preliminary experiments (reactions at 7 mM Mg^{2+}, pH 8.0, 37°C) full-length product was synthesized in about six minutes (approximately 1250 nucleotides/min) (data not shown).

Not only were the elongation and initiation rates affected by changes in the reaction conditions, but the nature of the product RNA synthesized also varied. When synthesized at 3 mM Mg^{2+}, the product RNA consisted of many size classes. Some full-length product RNA as well as a large amount of subgenome-sized product RNA was synthesized (Fig. 14; Fig. 12, lanes 1 and 3). Some of the small RNA migrated as discrete bands in the agarose gels (Fig. 14; Fig. 12, lanes 1 and 3). The majority of small product RNA could not be chased into full-length molecules either by longer incubation times or by increasing the Mg^{2+} concentration to 7 mM once the reaction was underway (J. B. Flanegan, unpublished results). When the reaction was carried out at 7 mM Mg^{2+}, most of the product was full-length.

The difference in the product synthesized at 3 mM Mg^{2+} and 7 mM Mg^{2+} was also evident when the product was analyzed in a nondenaturing agarose gel (Fig. 11B). The product RNA synthesized at 3 mM Mg^{2+} migrated in a region of the gel between the poliovirus dsRNA and ssRNA markers, with discrete bands often being apparent (Fig. 11B, lane 4b). The product RNA that was synthesized in a reaction containing 7 mM Mg^{2+}, however, migrated as a single band a little slower than dsRNA (Fig. 11B, lane 3b). When electrophoresed in a CH_3HgOH-agarose gel, the largest product RNA from

Figure 13.

Measurement of elongation rates for the *in vitro* polymerase reaction. Elongation rates were determined by electrophoresing labeled product RNA in a CH_3HgOH-agarose gel after various periods of reaction time. (A) The gel was autoradiographed, and the size of the largest labeled product RNA was measured and plotted as a function of reaction time. The average elongation rate for all time points was determined. (B) The same gel was stained with ethidium bromide. Stained markers were poliovirus dsRNA and 18 S and 28 S HeLa ribosomal RNAs.

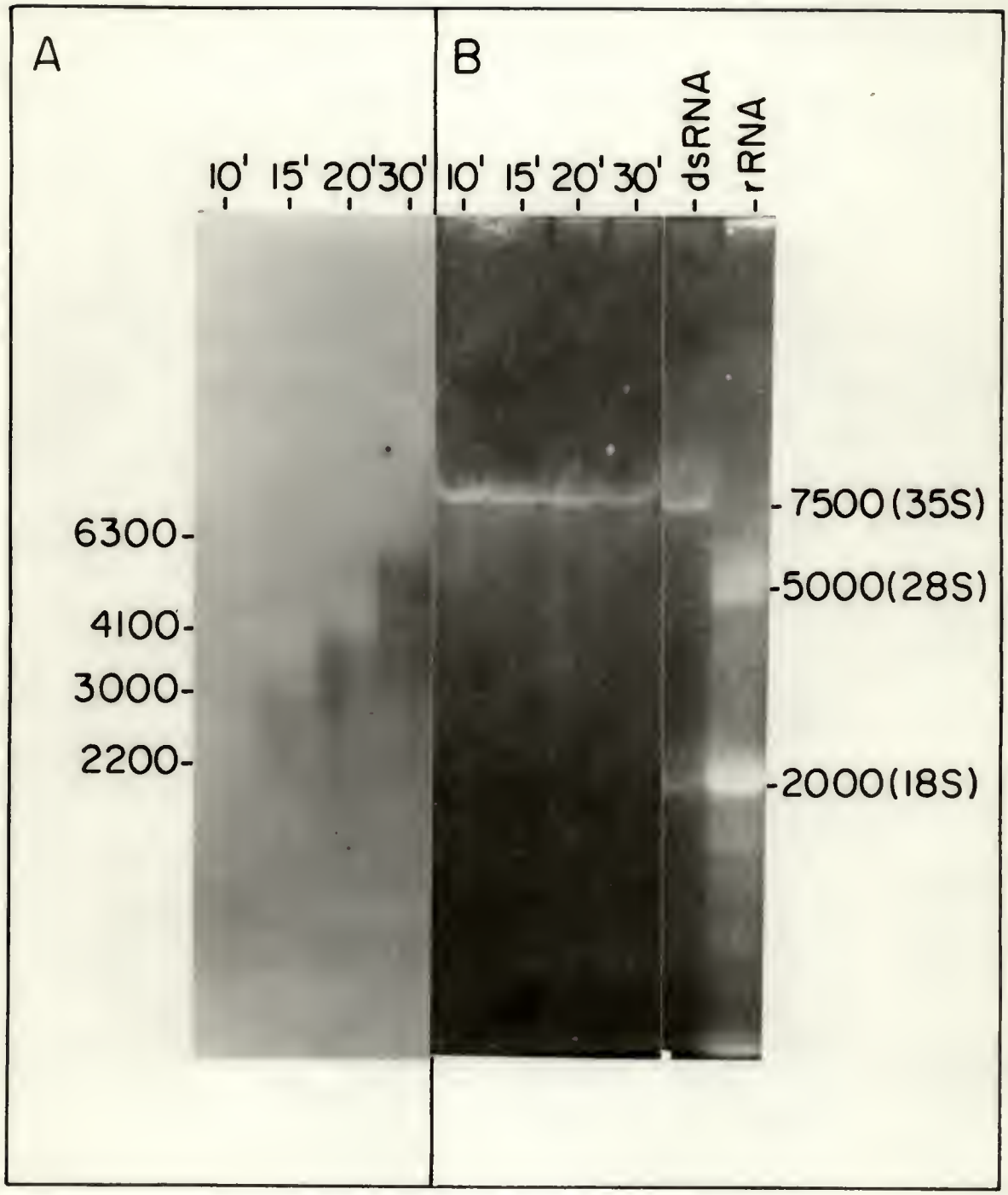

A
B
10' 15' 20' 30'
10' 15' 20' 30'
-dsRNA
-rRNA
6300-
4100-
3000-
2200-
-7500(35S)
-5000(28S)
-2000(18S)

Figure 14.

Effect of pH and Mg^{2+} concentration on the synthesis of product RNA. Labeled product RNA which was synthesized at pH 7 or pH 8 and 3 mM Mg^{2+} or 7 mM Mg^{2+} was analyzed in a Ch_3HgOH-agarose gel after various reaction times. Chain lengths were calculated based on the sizes of the 35 S, 38 S, and 18 S marker RNAs which were detected in the gel by staining.

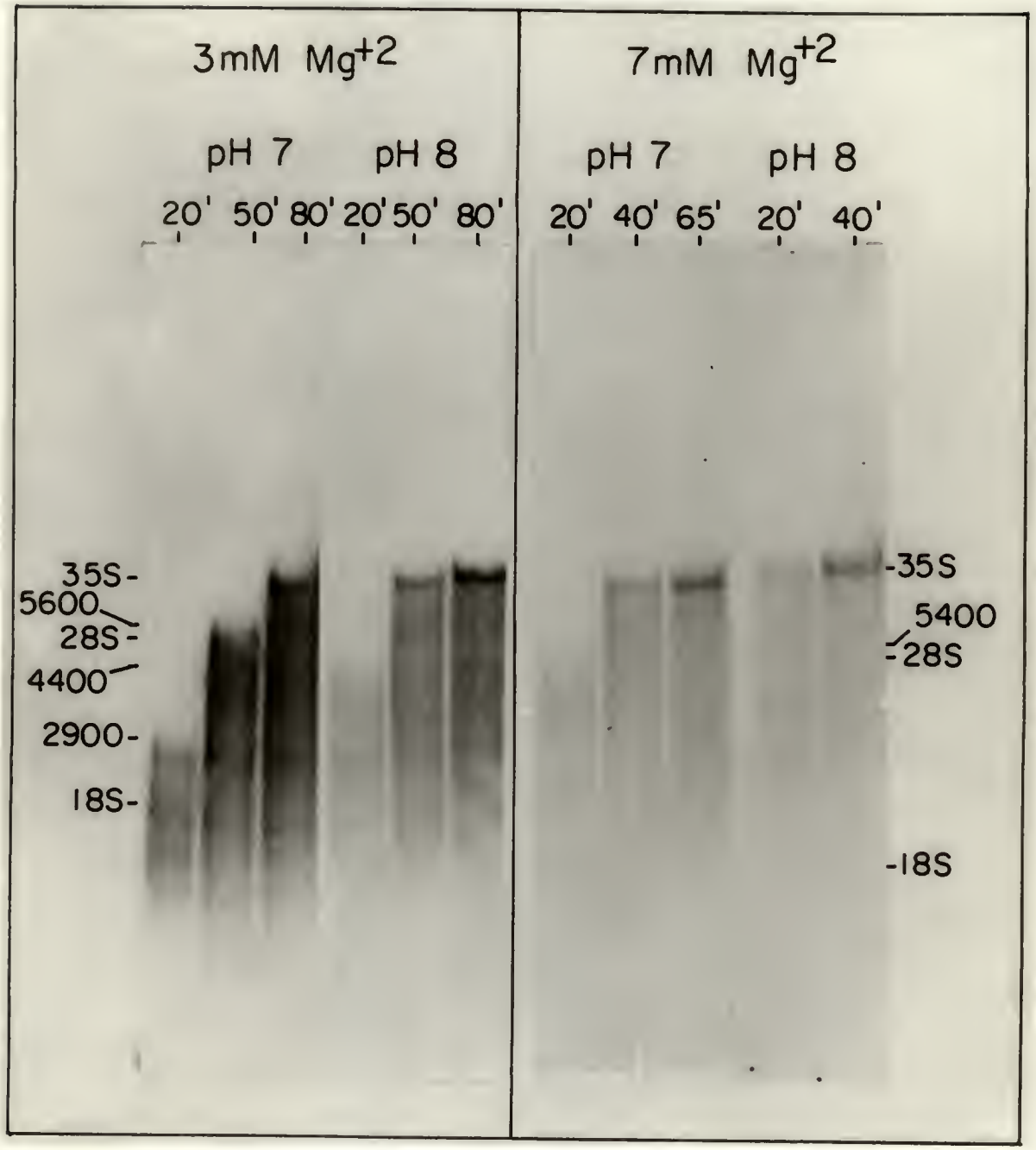

3mM Mg+2
pH 7
pH 8
20' 50' 80' 20' 50' 80'
35S-
5600
28S-
4400
2900-
18S-
7mM Mg+2
pH 7
pH 8
20' 40' 65' 20' 40'
-35S
5400
-28S
-18S

Figure 15.

Quantitative analysis of the rates of elongation and incorporation in the *in vitro* polymerase reaction. (A) Elongation rates were determined as shown in Fig. 13. (B) Incorporation rates for the same reactions were measured as the amount of [α^{32}P]GMP incorporated into acid-insoluble material during a given time period.

A
7mM Mg+2
pH 8.0
3mM Mg+2
pH 8.0
3mM Mg+2
pH 7.0
Length of product RNA
(nucleotides x10-3)
Reaction time (minutes)
B
3mM Mg+2
pH 7.0
3mM Mg+2
pH 8.0
7mM Mg+2
pH 8.0
[32P] GMP incorporated (pmole x10)
Nucleotides incorporated x10-3
Reaction time (minutes)

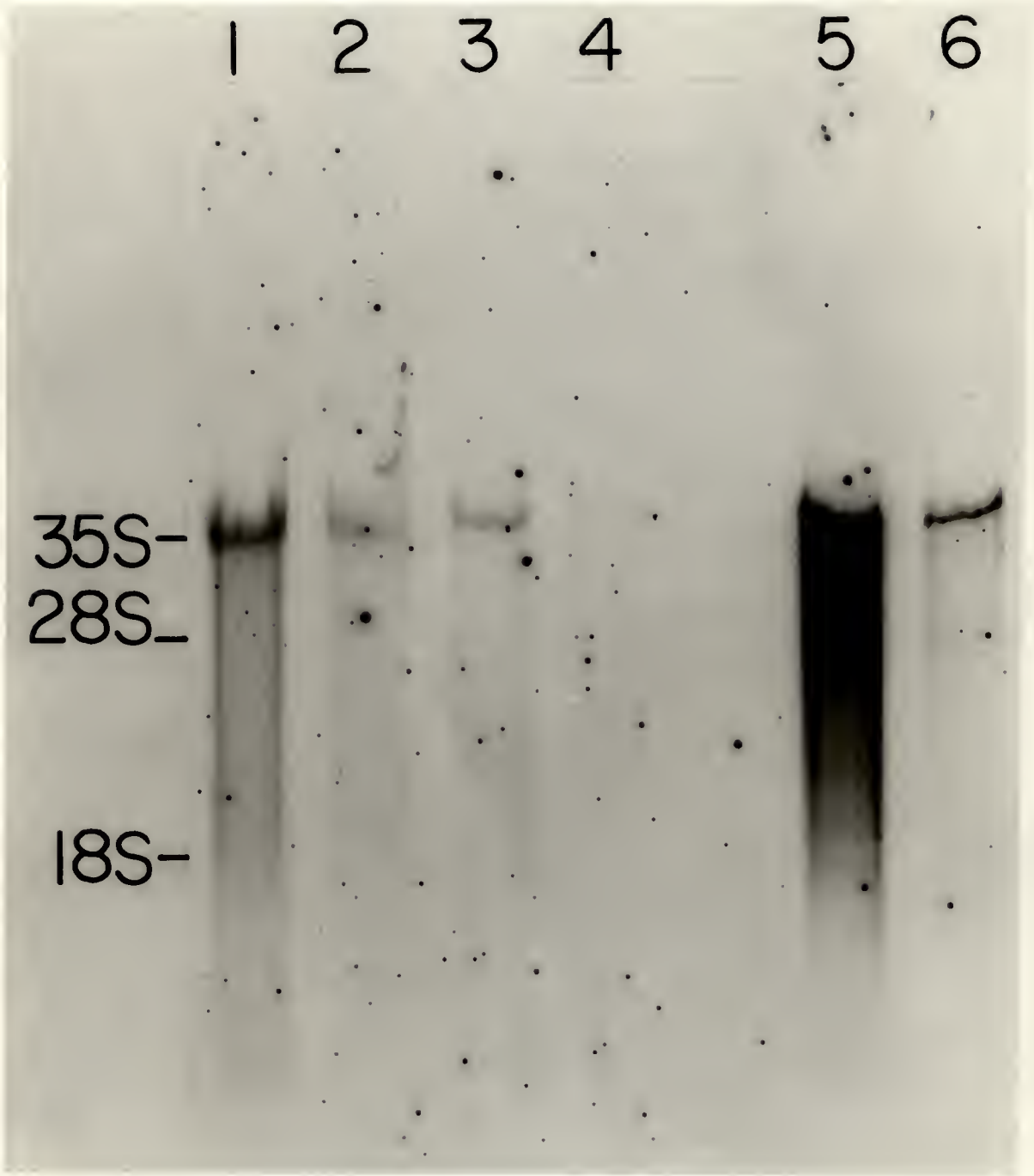
both reactions migrated with the same mobility as poliovirus dsRNA and 35 S RNA (Fig. 16, lanes 5 and 6). The mechanism and rates of synthesis of product RNA in the *in vitro* reaction, therefore, were clearly different depending upon the conditions of the reaction.

Oligo(U) Linkage to Product RNA

It was important to determine if the oligo(U) used in the *in vitro* polymerase reaction had become covalently linked to the product RNA. Product RNA was synthesized in the presence of unlabeled nucleotides and [^{32}P]oligo(U). The product was then analyzed on a 1% agarose gel containing CH_3HgOH. The presence of full-length molecules which were radiolabeled (Fig. 16, lanes 1-4) indicated that the [^{32}P]oligo(U) had become covalently associated with the product RNA in the reaction. The product which had been labeled with [^{32}P]oligo(U) appeared to be similar to that synthesized in the presence of [α^{32}P]GTP and unlabeled oligo(U). This was true whether synthesized at either 3 mM Mg^{2+} (Fig. 16, lanes 1, 3 and 5) or 7 mM Mg^{2+} (Fig. 16, lanes 2, 4 and 6). In addition, the [^{32}P]oligo(U)-labeled product RNA was the same when synthesized by the fraction III polymerase (Fig. 16, lanes 1 and 2) and by the fraction V enzyme (Fig. 16, lanes 3 and 4). The product RNA was not labeled by an artifactual kinase reaction that involved the small amount of [γ^{32}P]ATP that remained in the [^{32}P]oligo(U) preparation. The [^{32}P]oligo(U) was separated from the contaminating [γ^{32}P]ATP by paper ionophoresis and was used in the *in vitro* polymerase reaction. In this case, the product RNA was again labeled as before (data not shown). The covalent linkage of the oligo(U)

Figure 16.

Covalent linkage of the oligo(U) primer to product RNA. Product RNA was synthesized in the presence of [^{32}P]oligo(U) and the four unlabeled nucleotides, and was electrophoresed in a CH_3HgOH-agarose gel. The [^{32}P]oligo(U)-labeled product RNA was compared with product RNA which had been labeled with [α^{32}P]GMP. Product RNA was analyzed as follows: lanes 1 through 4, product RNA labeled with [^{32}P]oligo(U); lanes 5 and 6, product RNA labeled with [α^{32}P]GMP. The product RNAs in lanes 1, 3, and 5 were synthesized at 3 mM Mg^{2+}, whereas product RNAs in lanes 2, 4, and 6 were synthesized at 7 mM Mg^{2+}. The fraction III (Table 1) polymerase was used to synthesize the product in lanes 1, 2, 5, and 6; and the fraction V polymerase (Table 1) was used for product on lanes 3 and 4. The positions of 18 S and 28 S HeLa ribosomal RNAs were determined by staining with ethidium bromide. Poliovirus ^{14}C-labeled dsRNA (20 S), which was electrophoresed in the same gel, was detected in a longer exposure.


1
2
3
4
5
6
35S-
28S-
18S-

to the product RNA indicates that the oligo(U) did indeed serve as a primer for the synthesis of the product RNA.

Discussion

We have shown that the purified poliovirus RNA polymerase synthesizes genome-length copies of virion RNA *in vitro*. In addition, the oligo(U) primer used in the reaction was covalently linked to the product RNA. These results suggest that the polymerase, using an oligo(U) primer, was able to initiate RNA synthesis at or near the 3'end of the template and carry out an elongation reaction which terminated at or near the 5'end of the template.

The reaction conditions significantly affected elongation rates, initiation rates, and the quality and amount of product RNA that was synthesized. At the optional magnesium ion concentration for incorporation (3 mM), most of the product RNA was smaller than genome-length and it consisted of many discrete size classes. It is possible that under these reaction conditions the polymerase stopped synthesis at specific points on the template which resulted in the production of these discrete size classes of product RNA. The product RNA was apparently still hybridized to the template after isolation from the reaction mixture because it migrated in a non-denaturing gel between poliovirus dsRNA and ssRNA markers. In addition, 90% of the product was resistant to RNase upon isolation from the reaction mixture.

At 7 mM Mg^{2+}, when the incorporation rate was low but the elongation rate was high, the majority of the product RNA synthesized by the polymerase was full-length. Only a very small amount of

subgenome-length product RNA was present and this was heterogeneous in size, appearing only as a light smear on a denaturing gel. The product of this reaction also appeared to be hybridized to the template RNA when it was isolated from the reaction and electrophoresed on a nondenaturing gel. In this case, however, the template-product complex migrated slower than poliovirus dsRNA. The reason for the slow migration of this complex is not yet understood. A replicative intermediate-like structure may be forming under these reaction conditions.

Other reaction conditions were also found to affect the synthesis of RNA in vitro. Lowering the pH of the reaction from 8 to 7 resulted in about a threefold reduction in the elongation rate, although the rate of incorporation remained the same. The change in pH did not seem to affect the size distribution of the product RNA that was synthesized. Temperature was shown to have an effect both on elongation rates and on the type of product RNA which was synthesized. An increase in the temperature of the 7 mM Mg^{2+} reaction from 30°C to 37°C resulted in a slightly faster elongation rate. When the temperature of the 3 mM Mg^{2+} reaction was 37°C, the majority of the product RNA was full length. The smaller product RNA, which was synthesized at 30°C, was no longer present (R. J. Rickles and J. B. Flanegan, unpublished results). Finally, the concentration of UTP in the in vitro reaction dramatically affected the elongation rate of the polymerase. When the UTP concentration was low (5 μM), the polymerase required more than 60 min to synthesize full-length product RNA (unpublished observation).

Under the optimal conditions that were tested in the *in vitro* reaction (pH 8.0, 7 mM Mg^{2+}, 37°C), full-length minus-strand RNA molecules were synthesized in about 6 min (unpublished results). *In vivo*, a complete plus-strand RNA molecule is synthesized in about 0.75 min (Baltimore, 1969). The *in vivo* rate for the synthesis of minus strands is not known. The maximum elongation rate of 1250 nucleotides per min achieved in this study approaches the *in vitro* elongation rate for the synthesis of eukaryotic DNA by polymerase-α (1700 nucleotides per min) (Fisher and Korn, 1977). Adenovirus DNA, which replicates with the aid of polymerase-α, is synthesized *in vitro* at a rate of 1200 nucleotides per min (Challberg and Kelley, 1979). The *in vivo* rate of eukaryotic DNA synthesis is about 1500-3000 nucleotides per min (Edenberg and Huberman, 1975), and adenovirus DNA is synthesized *in vivo* at a rate of 1150 to 2100 nucleotides per min (Bodnar and Pearson, 1980). Whether the rate of RNA synthesis *in vitro* by the purified RNA polymerase is comparable to the *in vivo* rate of poliovirus minus-strand RNA synthesis remains to be determined.

We have shown in this study that the purified polymerase requires a primer for the synthesis of RNA *in vitro*. The polymerase will also make full-length copies of other polyadenylated templates in the presence of an oligo(U) primer (D. Tuschall and J. B. Flanegan, unpublished results). These results suggest that the *in vivo* specificity of the poliovirus RNA polymerase for poliovirus RNA may be conferred by the initiation reaction.

The requirement of the polymerase for a primer *in vivo* has not been established. Some preparations of polymerase from poliovirus-infected cells (Dasgupta *et al.*, 1979, and our unpublished results)

and from FMDV-infected cells (Lowe, P. A., and F. Brown, Virology, in press) have had a low level of polymerase activity in the absence of an oligo(U) primer. These enzyme preparations, however, were not highly purified, and may have contained other factors which aided in the initiation of RNA synthesis.

All DNA polymerases discovered thus far require a primer, usually RNA, for activity (Kornberg, 1980). Adenovirus DNA may replicate in a unique fashion by utilizing a protein linked to a nucleotide as a primer (Challberg et al., 1980; Stillman, 1981; Ikeda et al., 1981). Evidence for this model, which has also been proposed for poliovirus RNA replication, is not yet complete. The DNA-dependent RNA polymerases appear to have the ability to initiate RNA synthesis de novo, usually with a purine triphosphate (Krakow et al., 1976). Likewise, the RNA bacteriophage replicases initiate RNA synthesis de novo using a purine and require additional proteins that are not necessary for elongation (reviewed by Blumenthal and Carmichael, 1979). Little is known about the mechanisms of initiation of RNA-dependent RNA synthesis among the animal viruses. The influenza virus transcriptase has recently been shown to utilize primers derived from host mRNAs for the synthesis of virus-specific transcripts (Krug et al., 1980; Dhar et al., 1980). It will be interesting to determine whether the mechanism for initiating RNA synthesis in the picornaviruses is similar to the known mechanisms which initiate the synthesis of other nucleic acids. Further investigation of the poliovirus polymerase activity in vitro will certainly contribute to an understanding of the molecular events which take place during picornavirus RNA-dependent RNA synthesis in vivo.

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BIOGRAPHICAL SKETCH

Terry was born on May 2, 1955, on Wake Island. Her parents are Betty I. Van Dyke and Harold Q. Van Dyke, and she has three older sisters, Janet, Barbara and Cathy. Terry attended public schools in Port Hueneme, California, and in Pocomoke City, Maryland. In June of 1973 she graduated from Pocomoke City High School and she entered Wake Forest University in August of the same year. After spending two years at Wake Forest with a major in biology, Terry transferred to the College of Arts and Sciences at the University of Florida. Her major there was interdisciplinary and consisted of studies in the fields of zoology, microbiology and biochemistry. She received a Bachelor of Science degree in June of 1977. Terry has been a graduate student in the Department of Immunology and Medical Microbiology, College of Medicine at the University of Florida since that time. While being supported by a training grant from the National Institutes of Health, Terry did her dissertation research in the laboratory of Dr. James B. Flanegan. After completion of the requirements for the degree of Doctor of Philosophy in June of 1981, she plans to pursue her studies, initially as a postdoctoral fellow. She will work first in the laboratory of Drs. Janet and Gary Stein at the University of Florida and then in the laboratory of Dr. Arnold Levine at the State University of New York at Stony Brook.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kenneth I Berns

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